IDENTIFICATION OF NITRATED PROTEINS THAT MAY PLAY A CRITICAL ROLE IN THE PATHOGENESIS OF METHOTREXATE INDUCED SMALL INTESTINAL DAMAGE IN THE RAT AND POSSIBLE WAYS TO PREVENT THE DAMAGE BY THE ADMINISTRATION OF NITRIC OXIDE SYNTHASE INHIBITOR

A THESIS TO BE SUBMITTED TO

THE TAMILNADU DR. M.G.R MEDICAL UNIVERSITY, CHENNAI

FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

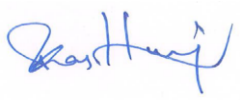
By

N. KASTHURI

Department Of Biochemistry
Christian Medical College
Vellore 632 002, Tamil Nadu
India

DECLARATION OF THE CANDIDATE

I, **N. Kasthuri** hereby declare that the thesis entitled "**Identification of nitrated proteins that may play a critical role in the pathogenesis of methotrexate induced small intestinal damage in the rat and possible ways to prevent the damage by the administration of nitric oxide synthase inhibitor**" is a record of research work done by me for the degree of **Doctor of Philosophy** under the supervision of **Dr. Premila Abraham**, Professor, Department of Biochemistry, Christian Medical College, Vellore – 632 002, India. This work has not been formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title of any other university. This thesis was written on the basis of regulations prescribed by The Tamil Nadu Dr. M.G.R. Medical University, Chennai.



N. Kasthuri, M.Sc.

Research scholar,

Department of Biochemistry,

Christian Medical College,

Vellore, India – 632 002.

Station : Vellore

Date : 01-04-2015

DEPARTMENT OF BIOCHEMISTRY
CHRISTIAN MEDICAL COLLEGE, VELLORE – 632002, INDIA

Telephone : 91-416-2284267, 2284458 **Fax**: 91-416-2262268 **E-mail**: biochemistry@cmcvellore.ac.in

MOLLY JACOB, M.B.B.S., M.D., Ph. D.
DHAYAKANI SELVAKUMAR, M.Sc., Ph.D.
PREMILA ABRAHAM, M.Sc., Ph.D.
MINNIE FAITH K., M.B.B.S., M.D.
JOE VARGHESE, M.B.B.S., M.D., D.N.B.

S.S. PRAKASH, M.B.B.S., M.D.
R. ANAND, M.B.B.S., M.D.

CERTIFICATE

This is to certify that the thesis entitled "**Identification of nitrated proteins that may play a critical role in the pathogenesis of methotrexate induced small intestinal damage in the rat and possible ways to prevent the damage by the administration of nitric oxide synthase inhibitor**" is based on the results of the work carried out by **Ms. N. Kasthuri** for the Ph.D. degree under my supervision. This work has not been submitted for any degree, diploma, associate ship, fellowship or other similar title of any other university.

Premila Abraham

Dr. Premila Abraham, M.Sc., Ph.D (Supervisor)
Professor of Biochemistry,
Christian Medical College,
Vellore, India – 632 002.

Dr. PREMILA ABRAHAM, Ph.D.
PROFESSOR IN BIOCHEMISTRY,
CHRISTIAN MEDICAL COLLEGE,
BAGAYAM, VELLORE - 632 002.

Station : Vellore

Date : 25-3-2015

DEPARTMENT OF BIOCHEMISTRY
CHRISTIAN MEDICAL COLLEGE, VELLORE – 632002, INDIA

Telephone : 91-416-2284267, 2284458 **Fax**: 91-416-2262268 **E-mail**: biochemistry@cmcvellore.ac.in

MOLLY JACOB, M.B.B.S., M.D., Ph. D.
DHAYAKANI SELVAKUMAR, M.Sc., Ph.D.
PREMILA ABRAHAM, M.Sc., Ph.D.
MINNIE FAITH K., M.B.B.S., M.D.
JOE VARGHESE, M.B.B.S., M.D., D.N.B.

S.S. PRAKASH, M.B.B.S., M.D.
R. ANAND, M.B.B.S., M.D.

CERTIFICATE

This is to certify that the thesis entitled "**Identification of nitrated proteins that may play a critical role in the pathogenesis of methotrexate induced small intestinal damage in the rat and possible ways to prevent the damage by the administration of nitric oxide synthase inhibitor**" is based on the results of the work carried out by **Ms. N. Kasthuri** for the Ph.D. degree under my supervision. This work has not been submitted for any degree, diploma, associateship, fellowship or other similar title of any other university.

Dhayakani Selvakumar

Dr. Dhayakani Selvakumar, M.Sc., Ph.D (Co-guide)
Professor of Biochemistry,
Christian Medical College,
Vellore, India – 632 002.

Dr. DHAYAKANI SELVAKUMAR, Ph.D.,
PROFESSOR OF BIOCHEMISTRY
DEPARTMENT OF BIOCHEMISTRY,
CHRISTIAN MEDICAL COLLEGE,
VELLORE -632 002. INDIA.

Station : Vellore

Date : 25-3-2015

Christian Medical College

VELLORE 632 002, INDIA

Dr. Alfred Job Daniel, Principal

Dr. Solomon Sathishkumar	Vice-Principal (UG)	**Dr. Joy Sarojini Michael**	Vice-Principal (PG)
Dr. John Mathew	Vice-Principal (AHS)	**Dr. Anna B. Pulimood**	Vice-Principal (HR)
Dr. Nihal Jacob Thomas	Addl.Vice-Principal (Research)	**Dr. Kuryan George**	Academic Officer

TO WHOMSOEVR IT MAY CONCERN

ETHICAL COMMITTEE CLEARANCE CERTIFICATE

IRB Min. No: 7460 dated 20-Apr-2011

The project titled "***Identification of nitrated proteins that may play a critical role in the pathogenesis of methotrexate induced small intestinal damage in the rat and possible ways to prevent the damage by the administration of nitric oxide synthase inhibitor***" has been reviewed by the Institutional Review Board of the Christian Medical College which considered its objective, study design, human research participant's protection and budget. This study has been approved for conduct at the Christian Medical College, Vellore by **Ms. N. Kasthuri**, Research fellow in Department of Biochemistry under the direction of **Dr. Premila Abraham**, Professor in Department of Biochemistry.

The IRB of Christian Medical College is registered and the institution holds FWA Number FWA00002210.

Best wishes

Dr. Alfred J Daniel

Chairperson (Research Committee) Institutional Review Board

Christian Medical College, Vellore 632 002

Chairperson (Research Committee) &
Principal
Christian Medical College
Vellore - 632 002, Tamil Nadu, India

Telephone : +91 (0) 416 228 4201/4202 **Principal** - princi@cmcvellore.ac.in **Telefax :** +91 (0) 416 226 2788

Vice Principal (UG) - princi.ug@cmcvellore.ac.in **Vice Principal (PG)** - princi.pg@cmcvellore.ac.in

Vice Principal (AHS) - princi.ahs@cmcvellore.ac.in **Vice Principal (HR)** - princi.hr@cmcvellore.ac.in

Vice Principal (Research) - research@cmcvellore.ac.in **Academic Officer** - princi.academic@cmcvellore.ac.in

INSTITUTIONAL ANIMAL ETHICS COMMITTEE
CHRISTIAN MEDICAL COLLEGE, VELLORE
(88/PO/RcBi/SL/1999/CPCSEA)

Dr. Alfred Job Daniel
Principal and Chairman
email: princi@cmcvellore.ac.in

Dr. Vinay Timothy Oommen
Secretary
email: vinayoommen@cmcvellore.ac.in

To whomsoever it May Concern

This is to certify that the animal studies done by **N. Kasthuri**, as part of her Ph.D thesis titled "**Identification of nitrated proteins that may play a critical role in the pathogenesis of methotrexate induced small intestinal damage in the rat and possible ways to prevent the damage by the administration of nitric oxide synthase inhibitor**", was approved by the Institutional Animal Ethics Committee of Christian Medical College, Vellore (IAEC approval Number 14/2009 dated 09/10/2009).

Dr. Vinay Timothy Oommen
Secretary, IAEC
Associate Professor,
Department of Physiology,
Christian Medical College,
Vellore, India – 632 002.

Place : Vellore

Date : 25.03.2015

ACKNOWLEDGEMENT

First and foremost, I offer my sincere gratitude to my guide and mentor, Dr. Premila Abraham, who supported me throughtout my research with her patience and knowledge. I attribute the level of my doctoral degree to her encouragement and effort without which this thesis would not have taken its shape.

I am grateful to my co-guide Dr. Dhayakani Selvakumar for her constant support during research period. I also express my heartfelt gratitude to Dr. Molly Jacob for her constant support and encouragement. I also thank Dr. Bina Isaac and Dr. Suganthy Rabi for their assistance in histology and histochemistry studies, valuable opinions and suggestions.

I would like to express my sincere thanks to my seniors Ms. Hemalatha, Mr. Viswa kalyan kolli, Mr. Nageshwaran, Mr. Arumugam, Ms. Abitha who were of continuous support to me during the extensive hours spent in the laboratory over the past five years. I would like to extend my gratitude to Mr. Sridhar, Mr. Isaac, Mr. Lalu, Mr. Kumaresan, Mrs. Punitha for their kind assistance.

I thank the Council of Scientific and Industrial Research, New Delhi, India for the confidence they had in me to extend the financial support to carry out the research work.

I am greatly indebted to the Principal, Christian Medical College, Vellore and The Tamil Nadu Dr. M.G.R. Medical University, Chennai for giving me an opportunity to pursue my doctoral studies in their institution.

I would be failing in my duty if I do not thank my parents, my siblings and my relations who directly or indirectly involved in making this thesis a success. They have been a great source of encouragement and support to me. This thesis is a result of all the minute sacrifices they made in order to see that I complete my work in time. In addition to these people, there are several others who stood as a support during the past five years of my doctoral work. I take immense pleasure in conveying my heartfelt gratitude to all of them.

Last, but not the least, I would like to acknowledge the contribution of the animals on whom I have carried out all my experiments. This thesis is a result of the sacrifice of their lives.

CONTENTS

LIST OF ABBREVIATIONS

3-NT	3-Nitrotyrosine
ACO	Aconitase
ADP	Adenosine di phosphate
AG	Aminoguanidine
AMP	Adenosine mono phosphate
ATP	Adenosine tri phosphate
BCS	Bathocuproine sulphaphonate di sodium salt
BSA	Bovine serum albumin
CA	Carbonic anhydrase
CASP3	Caspase 3
CASP9	Caspase 9
CAT	Catalase
CBB	Coomassie brilliant blue
CDNB	1-chloro-2,4-dinitrobenzene
CK	Creatine kinase
COX2	Cyclooxygenase 2
CYTC	Cytochrome C
CYTC.OX	Cytochrome C oxidase
CYTO	Cytosol
DAB	Diamino benzoic acid
DAPI	4′,6-diamidino-2-phenylindole
DCPIP	Dichlorophenol indophenol
DMSO	Dimethyl sulfoxide

DNA	Doxyribo nucleic acid
DNPH	Dinitro phenyl hydrazine
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
ETC	Electron transport chain
F1ATPase	Fragment 1 Adenosine triphosphatase
GDH	Glutamate dehydrogenase
GIT	Gastrointestinal toxicity
GPO	Glutathione peroxidase
GSH	Glutathione (reduced form)
GST	Glutathione S transferase
H_2O_2	Hydrogen peroxide
HEPES	[N-(2-Hydroxy ethyl)]piperzine-N(2-ethane sulphonic acid)
HO1	Hemeoxygenase 1
HOMO	Homogenate
HSP70	Heat shock protein 70
IF	Immunofluorescence
IHC	Immunohistochemistry
IkB	Inhibitory kappa B
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
KCN	Potassium cyanide
$MgCl_2$	Magnesium chloride
MICR	Microsomes
MITO	Mitochondria

MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
$MnCl_2$	Manganese chloride
MnSOD	Manganese superoxide dismutase
MPO	Myeloperoxidase
MTT	3-(4,5-dimethylthiazole-2yl)-2,5-diphenyl tetrazolium
MTX	Methotrexate
NAD	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NFκB	Nuclear factor kappa B
NO	Nitric oxide
NTY	Nitrotyrosine
NUCl	Nucleus
PARP	Poly ADP ribose polymerase
PLA_2	Phospholipase A2
PNPA	p-Nitrophenyl acetate
PON	Peroxynitrite
PTM	Post translational modification
PTN	Protein tyrosine nitration
PVDF	polyvinylidene difluoride
RNA	Ribo nucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

RT-PCR	Reverse transcriptase – Polymerase chain reaction
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TCA	Trichloro acetic acid
TEA	Triethanolamine
TNF-α	Tumor necrosis factor alpha
TRIS	Tris (hydroxyl methyl) aminomethane
TTFA	2- theonyl trifluoroacetone
WB	Western blot

CHAPTER – 1

Introduction

Chemotherapy and radiation therapy are the most widely used interventions for the treatment of cancer and chemotherapy is one of the principal modes of the treatment of cancer patients. Although chemotherapy is employed to improve the patient's quality of life, it is associated with several side effects. Severe adverse reactions result in patient morbidity and mortality. In addition, it also contributes to economic ramifications of the affected patient.

Mucositis is a debilitating, dose-limiting, and costly side effect of cancer therapy. Mucositis is the term used to describe the damage caused to mucous membranes of the alimentary tract by radiation and chemotherapy, in particular with drugs affecting DNA synthesis (such as fluorouracil, methotrexate, and cytarabine) (1-2). Mucositis occurs in 40% of cancer patients after standard doses of treatment and in almost 100% of patients treated with high doses of chemotherapy (1) and can affect the entire gastrointestinal tract causing discomfort, nausea, vomiting, bloating, diarrhoea, ulceration, bleeding and in some cases result in septicaemia (2).

The epithelium in the small intestine is extremely sensitive to cytostatic drug treatment, since it is proliferating rapidly. The loss of intestinal epithelial integrity causes pain and ulceration, vomiting, bloating, diarrhoea, symptoms of malabsorption, and an enhanced risk of bacteremia. The clinical presentations depend on the area of the alimentary tract affected. Mucositis limits the patient’s ability to tolerate chemotherapy or radiation therapy (leads to dose reduction or prevention of continuation of selected cancer therapies), prolongs hospital stay, increases re-admission rates, compromises the patient’s nutritional status, affects the patient’s quality of life, and is occasionally fatal. Majority of patients suffering from mucositis, the rely on pain relief as their only treatment option (3).

Methotrexate (MTX), an analogue of folic acid is the most commonly used anti-metabolite agent in clinical oncology practice. It is effective in the treatment for malignant acute leukemia, non-Hodgkins lymphoma, carcinoma, primary CNS lymphoma, squamous cell carcinoma, choricarcinoma, adenoma and melanoma (4) as well as several non-malignant inflammatory diseases including wegeners granulomatosis, dermatomyositis, sarcoidosis, alkylosing spondylitis, skin diseases, behcet disease, multiple sclerosis, uvetis, systemic vasculitis, idiopathic hypertrophic cranial pachemeningitis, neoplastic meningitis, myopathy, ectopic pregnancy conditions and ulcerative colitis (5-6). It is recommended as a first line disease modifying treatment for rheumatoid arthritis (7-8), psoriatic arthritis, and psoriasis (9-10) and for both the induction and maintenance of remission in Crohn's disease (6, 11-12).

The efficacy of MTX is often limited by severe side effects such as bone marrow suppression, oral and intestinal mucositis. The adverse effects of MTX, such as renal or liver impairment, testicular damage, neurotoxicity, teratogenecity have also been reported in patients (13-17).

Gastrointestinal mucositis is one of the most severe and debilitating side effects of MTX treatment at both low and high dose of methotrexate (18) . MTX induced small intestinal damage incorporate intestinal lesions, malabsorption (19), jejunal crypt cell abnormalities (20) and epithelial changes, bleeding and ulceration (21-22). The use methotrexate in anticancer treatments may severely impair intestinal epithelial function and therefore constitutes a dose limiting factor in treatment schedules (20). Like other chemotherapeutics, it induces diarrhea and anorexia, accompanied by malabsorption, malnutrition, and dehydration. MTX induced enteritis

is characterized by small intestinal villus atrophy and loss of enterocytes resuting in malabsorption syndrome, leading to poor absorption of nutrients and diarrhea (3).

To date, there is no definitive prophylaxis or treatment for MTX induced mucositis. This is due in part to the lack of understanding of its pathogenesis and its impact on intestinal structure and function. Although many studies have been carried out the exact mechanism of gastrointestinal toxicity of MTX is not completely understood. Therefore, treatment / prevention of MTX induced gastrointestinal mucositis is still elusive.

Elucidating the mechanisms that underlie MTX induced mucosal injury would lead to treatments targeted to preventing the loss of mucosal barrier functions that lead to these debilitating and painful side effects of this antineoplastic therapy. Therefore, our research focus is to elucidate the mechanism of MTX enteritis. Research has been performed on the effects of cytostatic drugs, including MTX, on the epithelium of the small intestine in animals. The use of animal experiments allows the analysis of the effects of aspecific drug over time in all regions of the intestine, which would be impossible to perform in humans. We have standardized a rat model of MTX induced GI mucositis in our laboratory and have carried out some studies which will help unravel the mechanism of MTX induced enteritis. We have demonstrated that mitochondrial damage, mitochondrial dysfunction, oxidative stress and nitrosative stress (tyrosine tyrosine nitration) contribute to MTX induced small intestinal damage (23-25).

The biological relevance of iNOS induction and protein tyrosine nitration in MTX induced small intestinal damage is not clear. Based on our earlier findings and those reported by others on the mechanisms of MTX induced small intestinal damage, the following pathway (Figure: 1.1) is hypothesized for the role of iNOS and its downstream targets in methotrexate induced small intestinal damage. In order to strengthen our hypothesis, we investigated whether the administration of aminoguanidine, a selective iNOS inhibitor protects rats against MTX induced enteritis

PROPOSED MECHANISM FOR THE ROLE OF INOS AND PEROXYNITRITE IN METHOTREXATE INDUCED SMALL INTESTINAL DAMAGE

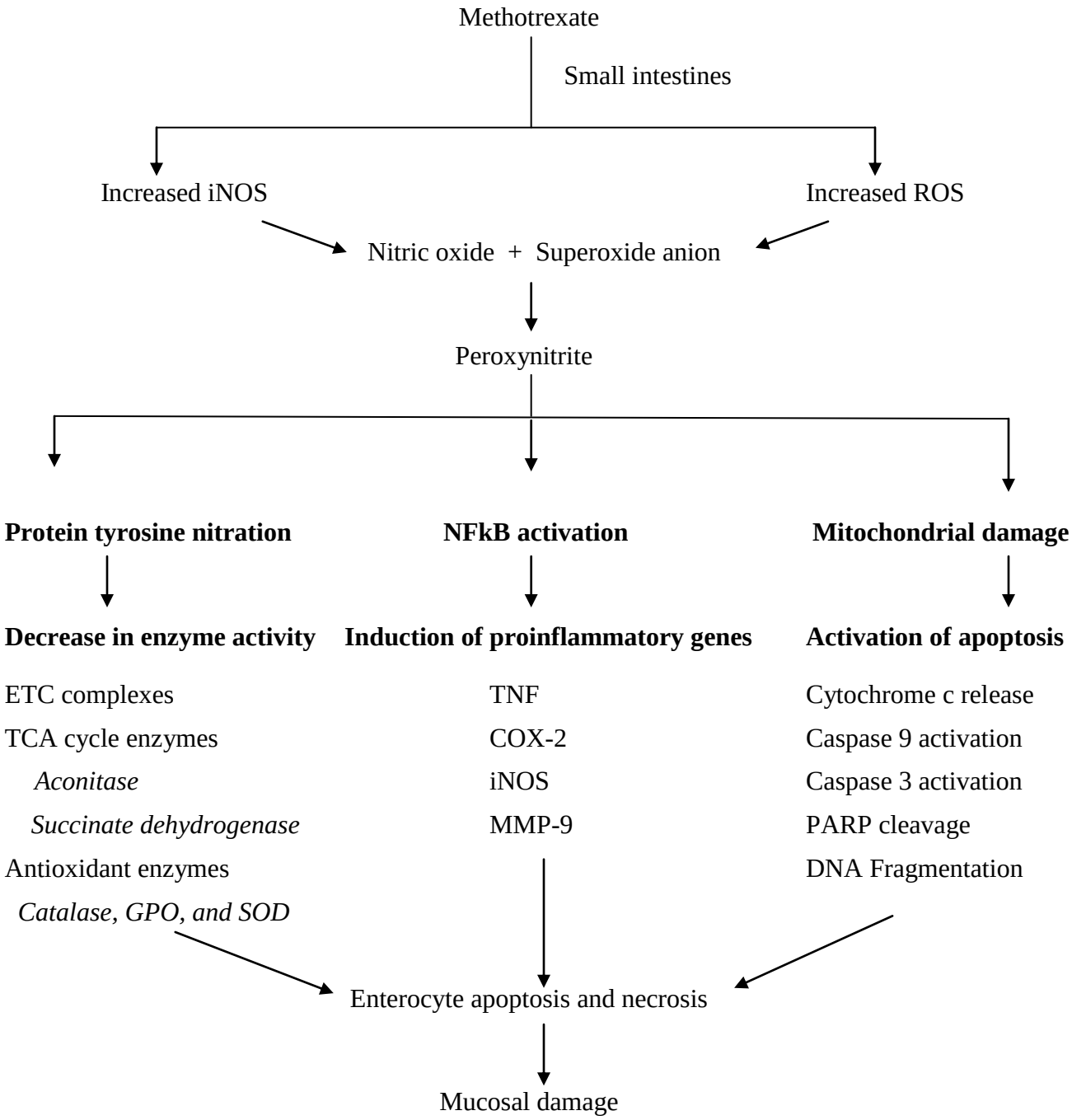


Figure: 1.1 - Mechanism hypothesized for methotrexate induced small intestinal damage.

CHAPTER – 2

Aims and Objectives

2.1 AIM:

The study was designed to investigate the roles of peroxynitrite (PON) induced protein tyrosine nitration, NFkappaB-iNOS-COX-TNFα signaling pathway and apoptotic pathway in methotrexate induced small intestinal damage and to evaluate the efficacy of aminoguanidine, a selective iNOS inhibitor in the prevention of the damage using rat model.

2.2 OBJECTIVES:

The specific objectives of the study were to:

1. Investigate the role of PON induced protein tyrosine nitration in MTX induced enteritis by the
    - analysis of the subcellular distribution of nitrated proteins
    - identification of the molecular weights of proteins that are nitrated
2. Determine the effect of MTX on the activities of enzymes that are established targets of peroxynitrite and are known undergo nitration during inflammatory conditions
3. Assess the role of NFκB-iNOS-COX-2-TNFα inflammatory signaling pathway in methotrexate induced enteritis
4. Investigate the role of apoptotic pathway in methotrexate induced enteritis
5. Investigate the protective effect aminoguanidine, a selective inhibitor of iNOS on methotrexate induced enteritis

CHAPTER – 3

Review of Literature

3.1 CHEMOTHERAPY

Chemotherapy and radiation therapy are the most widely used interventions for the treatment of cancer and chemotherapy is one of the principal modes of the treatment of cancer patients (26). It was first used to treat advanced lymphoma in the late 1940s after it became known that the use of mustard gas in the World War I caused leukopenia (27). Shortly after the World War II, it was also found that folic acid stimulates the proliferation of acute lymphoblastic leukaemia cells and antagonistic analogues to folic acid, first aminopterin and then amethopterin (now known as methotrexate) induced the remission in children with acute lymphoblastic leukaemia (28).

Cancer treatment is targeted at its proliferation potential and its ability to metastasise; hence, the majority of chemotherapy drugs take advantage of the fact that cancer cells divide rapidly (29). Chemotherapy agents can be divided into several categories based on the factors such as how they work, their chemical structure, and their relationship to another drug. The most important categories of chemotherapeutics include alkylating agents (e.g., cyclophosphamide, ifosfamide, melphalan, busulfan), antimetabolites (e.g., 5-fluorouracil, capecitabine, methotrexate, gemcitabine), antitumourantibiotics (e.g., daunorubicin, doxorubicin, epirubicin), topoisomerase inhibitors (e.g., topotecan, irinotecan, etoposide, teniposide), and mitotic inhibitors (e.g., paclitaxel, docetaxel, vinblastine, vincristine) (30).

3.1.1 Factors Affecting the Effectiveness of Chemotherapy

Effectiveness of chemotherapy depends on various factors, including properties of cancer cells. If tumour is hypoxic or mitochondrial function is severely compromised, or the number of mitochondria within the cancer cell is low, chemotherapy will be of limited value. The

microscopic form of tumour is much more successfully treated than macroscopic cancer. The other factors that can affect chemotherapy include the number of chemotherapy cycles; administrating polychemotherapy *versus* monotherapy etc. Polychemotherapy may be more active than single agent, whereas the order of administration of drugs as well as their time schedule is also important. Combining drugs with different modes of action may lead to enhanced or even synergistic antitumour effects without injuring the host, multitargeted approach targeting both the cancer cell and its microenvironment might increase the treatment efficiency and the phase of the circadian cycle (31).

3.1.2 Typical Side Effects of Chemotherapy

Although chemotherapy is employed to improve the patient's quality of life, it is associated with several side effects. Severe adverse reactions result in patient morbidity and mortality. In addition, they also contribute to economic ramifications of the affected patient (31). Although the desired goal of chemotherapy is to eliminate the tumour cells, normal haematopoietic cells, intestinal epithelial cells, and hair matrix keratinocytes are often susceptible to the toxic effects of anticancer agents (32), leading to many adverse side effects in multiple organ systems including bone marrow, liver, kidney, nervous system, lungs and GIT (33). (Figure: 3.1).

Knowing how the chemotherapy agent works is important in predicting its side effects. For instance, treatment with alkylating agents and topoisomerase II inhibitors increases the risk of secondary cancer (acute leukaemia); anthracyclines (like doxorubicin) induce cardiotoxicity; and mitotic inhibitors have the potential to cause peripheral nerve damage (34).

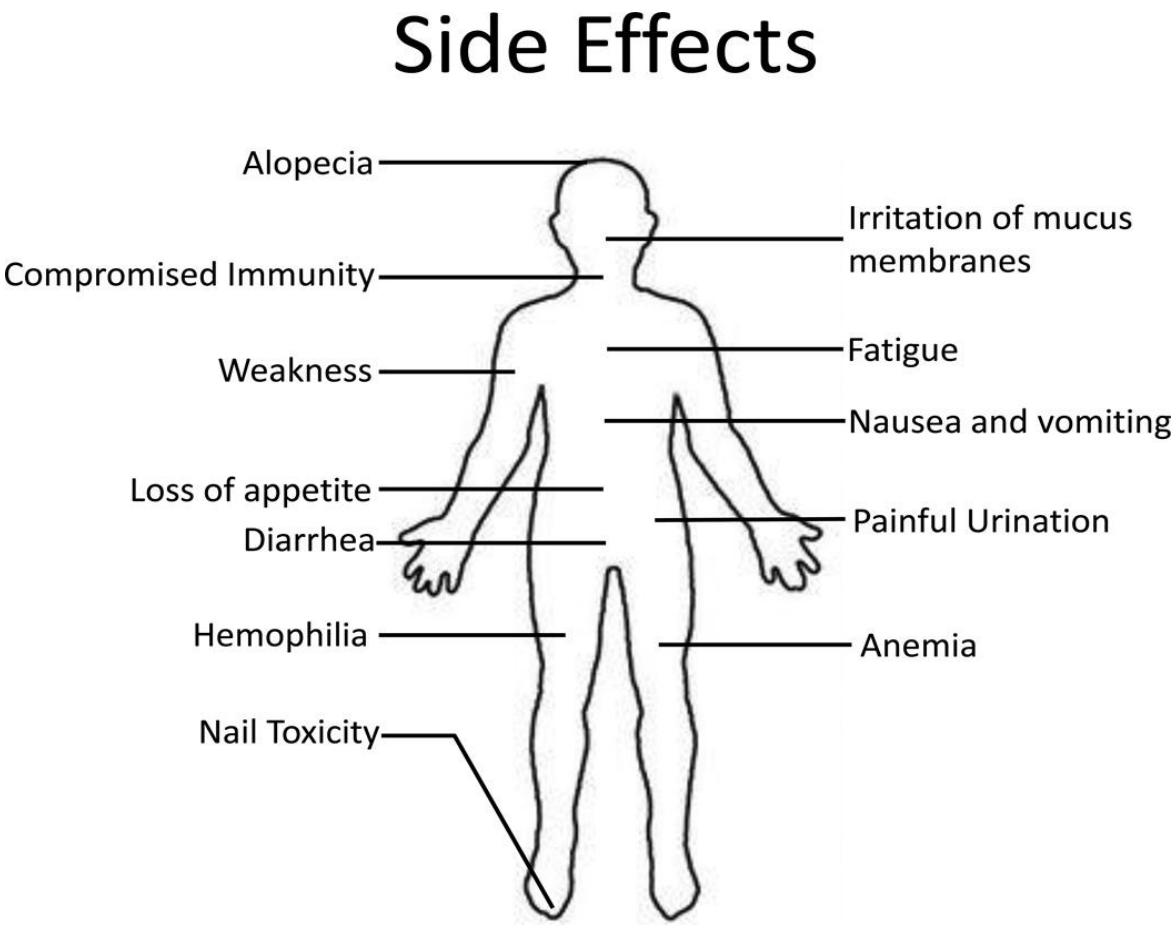


Figure: 3.1 - Side effects of anti-cancer agents (33)

The most common acute complaints of cancer patients undergoing cytotoxic therapy are fatigue, nausea, vomiting, malaise, diarrhoea, mucositis, pain, rashes, infections, headaches, and other problems. Furthermore, ulceration of the GIT is commonly associated with a high risk of systemic infection which poses a threat to patient health. It must be appreciated that malnutrition is the reason why majority of the cancer patients die (35). The most common long-term health problems of adjuvant chemotherapy include poor memory and concentration, visual deterioration, musculoskeletal complaints including early onset osteoporosis, poor sleep patterns, skin changes, sexual dysfunction, and chronic fatigue (33) (Figure: 3.1). Such debilitating effects are a major clinical problem, whereas the toxicity often limits the usefulness of anticancer agents.

3.2 METHOTREXATE

3.2.1 Introduction

Methotrexate (MTX), also known as amethopterin, is an antimetabolite utilized as chemotherapeutic drug for diverse malignancies. Methotrexate is a structural analogue of folic acid. The cytotoxicity of MTX in small intestine is mediated by the inhibition of folate reductase and DNA synthesis and also purine and protein synthesis. The primary target of MTX is dihydrofolate reductase enzyme which catalyze the conversion of folate and 7,8 dihydrofolate to 5,6,7,8 tetrahydrofolate (36).

3.2.2 Chemistry

Methotrexate is 4-amino-4-deoxy-N-10-methylpteroylglutamic acid with a molecular weight of 454 daltons. Its molecular structure differs from folic acid only in that folic acid has a hydroxyl

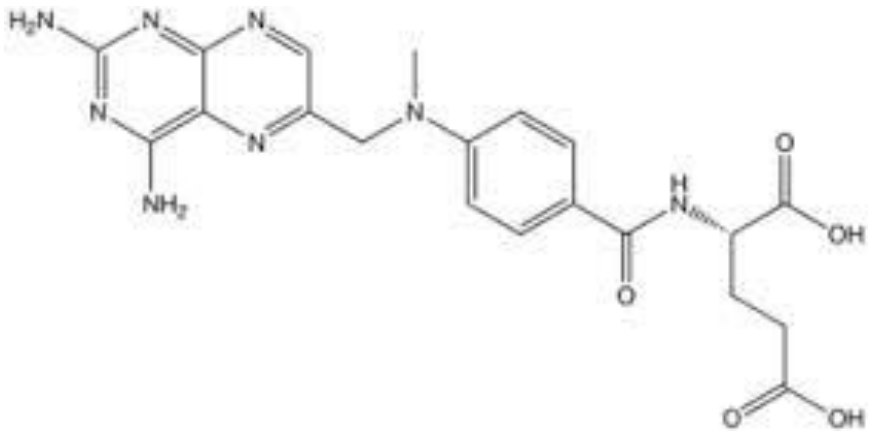


Figure: 3.2 - Structure of Methotrexate (37)

group in place of the 4- amino group on the pteridine ring and there is no methyl group at the N^{10} position (37). (Figure: 3.2)

3.2.3 MTX as therapeutic agent

Methotrexate is an effective treatment for malignant diseases like acute leukemia, non-Hodgkins lymphoma, carcinoma, primary CNS lymphoma, squamous cell carcinoma, choricarcinoma, adenoma and melanoma (4) as well as several non-malignant inflammatory diseases including wegeners granulomatosis, dermatomyositis, sarcoidosis, alkylosing spondylitis, skin diseases, behcet disease, multiple sclerosis, uvetis, systemic vasculitis, idiopathic hypertrophic cranial pachemeningitis, neoplastic meningitis, myopathy, ectopic pregnancy conditions and ulcerative colitis (5-6). It is recommended as a first line disease modifying treatment for rheumatoid arthritis (7-8), psoriatic arthritis, and psoriasis (9-10) and for both the induction and maintenance of remission in Crohn's disease (11). Methotrexate may be used as monotherapy or in combination with other drugs, including oral agents. In some disorders methotrexate is not only disease altering but also life saving (38-39).

3.2.4 Mechanism of action

MTX competitively inhibits the enzyme dihydrofolate reductase which catalyses the conversion of dihydrofolate to tetrahydrofolate (40). MTX has high affinity to bind to DHFR and inhibits folate synthesis which is essential for the de novo synthesis of thymidine, required for DNA synthesis. The folate is also needed for purine synthesis, thus MTX inhibits purine biosynthesis. Therefore, MTX inhibits DNA, RNA, and eventually protein synthesis (41). Biochemical mechanism of action of MTX modulating inflammation are inhibition of transmethylation reactions and promotion of adenosine release (42). The *in vivo* effect of MTX is also mediated

by decrease in cell proliferation, inhibition of T cell activation, suppression of cellular adhesion molecules, and deactivation of enzymes required for immune system (43).

3.2.5 MTX bioavailability

Oral, subcutaneous or intramuscular of MTX have similar bioavailability at lower dose. Around 70% of the drug is absorbed both in fasting and after food (44). During higher dose of MTX the bioavailability is decreased in oral dose when compared to intramuscular and subcutaneous dose (45). It is suggested that splitting higher dose weekly improves the bioavailability of oral administration. This variation in high dosage is due to limitation in gastrointestinal tract absorption. However, the superior MTX bioavailability is reported by parenteral administration than oral administration (46).

3.2.6 MTX metabolism

MTX enters into the cell by reduced folate carrier PCFT/HCP-1 (Proton coupled folate transporter / Heme carrier protein -1). Polyglutamation of MTX inside the cell is catalyzed by folyl – polyglutamate synthetase. The polyglutamation process is slow and takes 27.5 weeks for to reach steady state. The transport of MTX and its metabolite are through passive, active or by facilitated diffusion method. The polyglutamate form of MTX is functionally important metabolite selectively retained in cell. Nearly 10% of MTX administered is oxidized to 7-hydroxy-methotrexate.

The active form of MTX, methotrexate polyglutamate is further cleaved by gamma glutamyl hydrolase enzyme in lysosome and act as a reservoir of MTX in liver. MTX is converted to

4-deoxy-4-amino-N10-methylpteroic acid by carboxypeptidase. MTX and its metabolites is cleared about 60-80% by kidney and the rest via bile. The estimated half life of MTX in serum is 7-10h where as half life of MTX in renal clearance is 12-24h. The half life of MTX elimination after intravenous administration is about 1-6h (47).

3.2.7 MTX dosage

Low dose MTX (LDMTX) around 20 mg/m^2 used for the treatment of rheumatoid arthritis and psoriasis where as High dose MTX (HDMTX) around 1000 mg/m^2 to 33000 mg/m^2 has become a standard chemotheraphy for cancers. HDMTX for osteosarcoma, lymphoma and leukemia is 3-15g/m2 for 6-24hr. Mean dose 18.2mg per week of MTX is used for Crohns disease and ulcerative colitis patient (48). High dose are given as split dose for long term.

3.2.8 MTX toxicity

The efficacy of MTX is often limited by severe side effects such as bone marrow suppression, oral and intestinal mucositis. The adverse effects of MTX, such as renal or liver impairment, testicular damage, neurotoxicity, teratogenecity have also been reported in patients (13-17). High dose toxicity of MTX includes gastrointestinal toxicity, neurotoxicity, haematologic toxicity, dermatological toxicity, pulmonary toxicity like shortness in breath, fatigue and fever, central nervous system toxicity like mood alteration, unpleasant sensation and memory impairment, severe myelotoxicity. Low dose MTX cause myelosuppression like severe pancytopenia and thrombocytopenia, leucopenia, neutropenia, common side effects include vomiting, nausea, diarrhea, alopecia, infections, oral mucosa lesion. (49)

3.2.9 Gastrointestinal side effects of methotrexate

Gastrointestinal mucositis is one of the most severe and debilitating side effects of MTX treatment at both low and high dose of methotrexate. Intestinal mucositis, bleeding and peptic ulcers are well-known gastrointestinal adverse effects of MTX. MTX induced small intestinal damage include intestinal lesions, malabsorption (19), jejunal crypt cell abnormalities (20), epithelial changes and ulceration (20). MTX induced enteritis is characterized by small intestinal villus atrophy and loss of enterocytes resuting in malabsorption syndrome, leading to poor absorption of nutrients and diarrhea (3).

3.3 GASTROINTESTINAL SYSTEM

Intestines are organs of digestive system involved in digestion of food, absorption of nutrients and also acts as defense barrier against ingested noxious agents and pathogens (50). The intestines include small intestine (20 feet long and an inch in diameter) which absorbs nutrients and minerals from food and the large intestine (5 feet long and 3 inches in diameter) which absorbs water. Small intestine is not only involved in the nutrient absorption and barrier function but is also responsible for signal recognition or transduction, maintenance of homeostatic processes and production of bioactive compounds. These functions are regulated by cytokines and hormones as well as diet. Even the low grade inflammation has its effect on gastrointestinal function by altering the process of digestion, absorption and barrier function promoting gastrointestinal dysfunction. Thus, proper modulation and function of small intestine is essential for good health. (Figure: 3.3)

The small intestine is a convoluted tube composed of three portions duodenum, jejunum and ileum. The proximal part of small intestine is duodenum involved in the fragmentation of the macromolecules into smaller compound using digestive enzymes. It contains Brunner's glands which produce mucus and bicarbonate that neutralize stomach acids. The absoption of iron takes place in the duodenum. The midsection of small intestine is jejunum that connects duodenum and ileum. It contains many folds plicae circulares, tiny finger like tissue villi and the epithelial cells with finger like projections called microvilli. Most of the nutrients like sugars, aminoacids and fatty acids are absorbed in the blood stream of jejunum. The terminal part of the small intestine is ileum that contains villi which are involved in the absorption of vitamin B_{12} and bile acids. It also contains the Peyers patches that establish immune surveillance in lumen of intestine and facilitate immune response. (Figure: 3.4)

Histology of small intestine is made up of four layers mucosa, submucosa, muscularis externa and serosa. The serosa is the outermost layer of flattened mesothelial cells with some connective tissue. The muscular coat with two layers of smooth muscles inner to the serosa in which individual muscle cells encircle the small intestine is muscularis externa which is essential for the segmentation and peristalsis. Submucosa is a layer of loose connective tissue which lies beneath the muscularis mucosa. The innermost layer of the small intestine is the mucosal layer which consists of numerous villi that are involved in the absorptive function of this organ. This mucosa is further layered in to outer thin layer of smooth muscle muscularis mucosae, a layer of connective tissue lamina propria and the innermost epithelium which is responsible for the process of digestion absorption and secretion. (Figure: 3.4)

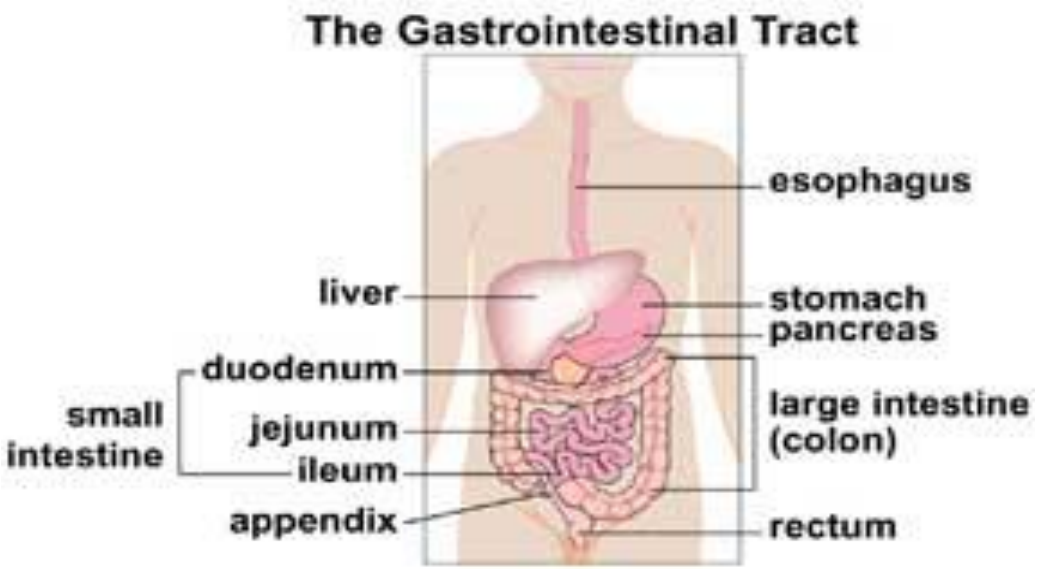


Figure: 3.3 - Schematic diagram of gastrointestinal tract (50)

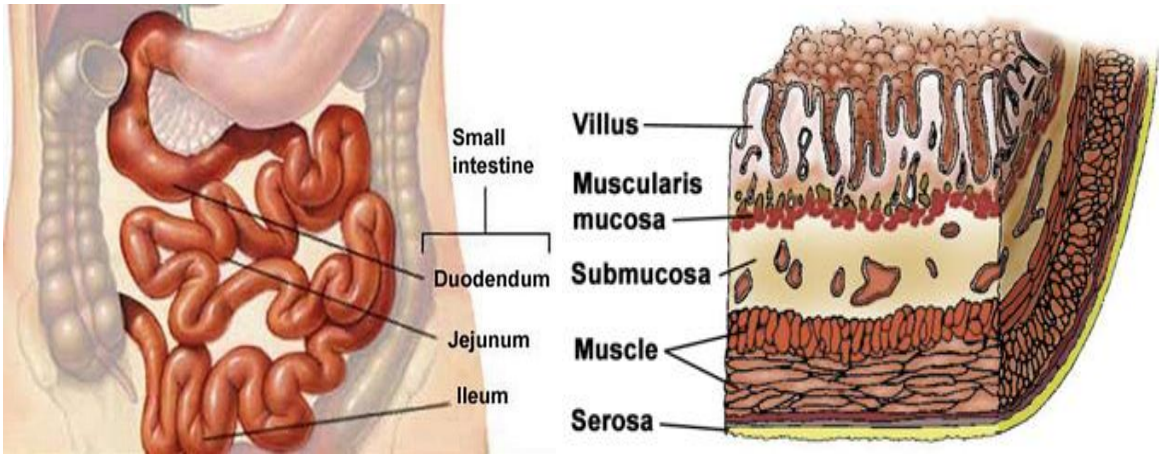


Figure: 3.4 - Schematic diagram of small intestine segments and layers (50)

3.4 GASTROINTESTINAL INFLAMMATION:

3.4.1 Inflammation:

Inflammation is a biological response of tissues when expose to harmful stimuli like pathogens and irritants. The signs of inflammation are redness, swelling, warmth and pain, also includes elevated cellular metabolism, release of mediators, and cellular influx. It is characterized as acute and chronic, based on the condition of the damaged cells and innate immune system plays a vital role. Acute inflammation is a normal protective process that heals incase of physical injury or infection. Chronic inflammation is a condition when the stimulus persists for longer time like days, months and years. Infiltration of macrophages and neutrophils characterize acute inflammation where as infiltration of plasma cells and T lymphocytes characterize chronic inflammation. Under normal conditions the inflammatory process is a self limiting process where as in disorders this develops subsequently and becomes chronic (51).

3.4.2 GI Inflammation:

The gastrointestinal toxicity mainly occurs due to the exposure of gastrointestinal tract to chemicals, foods, concentrated acids and pathogens. The other side of the toxicity is due to the disruption of gastrointestinal defense mechanism by metals, chemicals, food allergens, radiation, pathogens and drugs (52). The gastrointestinal disorders are caused mainly by inflammation, infection and malignancies. The inflammatory condition of gastrointestinal tract includes gastritis, colitis and enteritis (53). Mucosal damage, gastrointestinal defense and subsequent repair is modulated by the inflammatory mediators (54). The inflammatory mediators of the gastrointestinal tract include nitric oxide, eicosinoids like prostaglandins and leukotrienes,

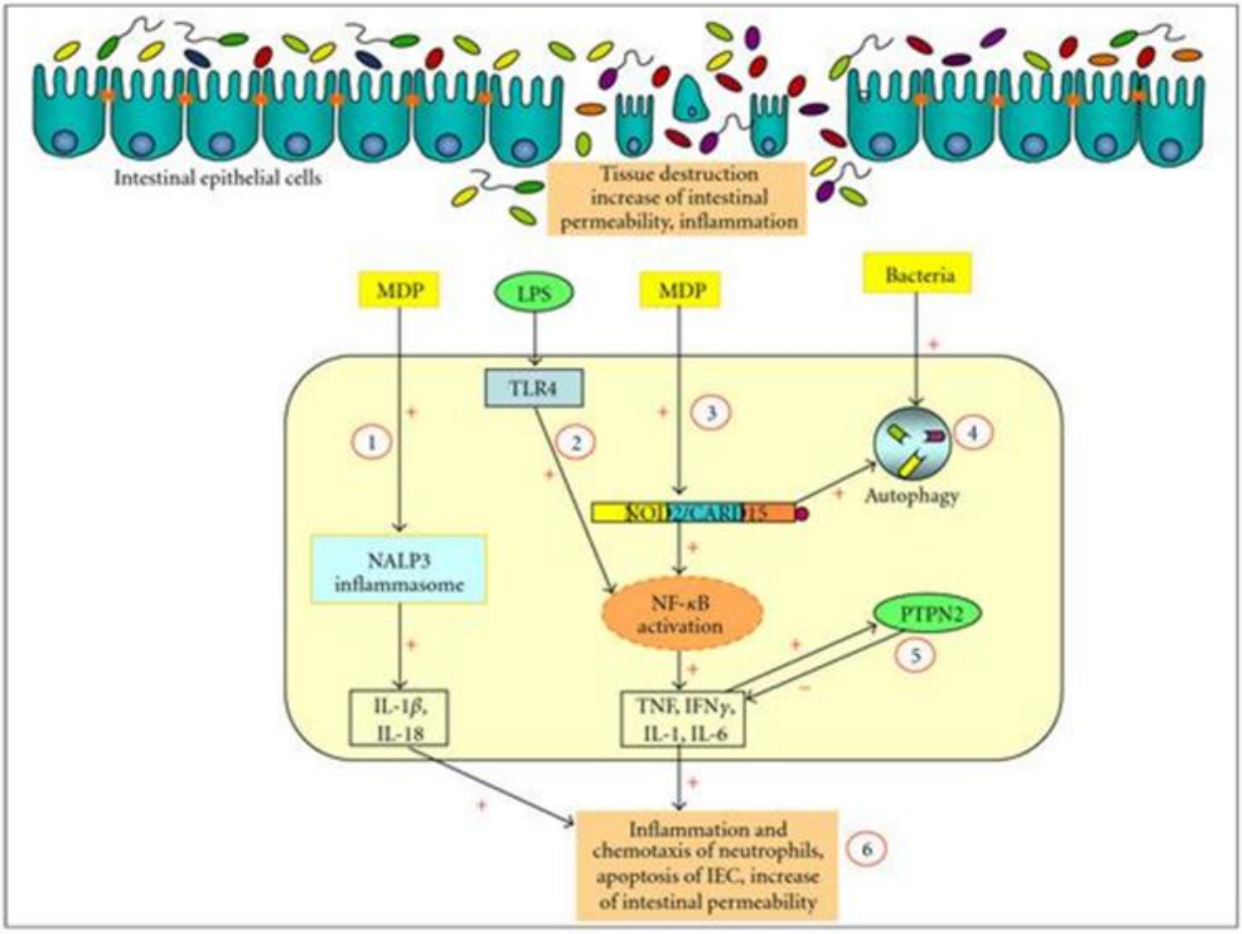


Figure: 3.5 – Mechanism of gastrointestinal inflammation (56)

cytokines like IL-1beta and TNF-α, and neuropeptides (55). The mechanism of gastrointestinal inflammation (56) is shown in Figure: 3.5

3.4.3 Levels of defense mechanism in GI inflammation:

The defense mechanism of mucosal inflammation is at different levels. First level is secretion of extramucosal components like acids, mucus, bicarbonate to reduce the ingested bacteria in small intestine. Second level is the barrier function of epithelium itself for diffusion of harmful agents. Third level is the response of mucosal microcirculation that increases the blood flow to prevent accumulation of harmful agents and to neutralize the toxic agents. Fourth level is the response of mucosal immune system that sense foreign materials and nullify by release of chemical mediators. Final level includes repairing system through growth of gastric glands, new blood vessels and reinnervation of mucosal nerves (57).

3.5 MUCOSITIS

The term "mucositis" specifically refers to the damage of mucous membranes throughout the entire gastrointestinal tract (GIT) following chemotherapy and radiotherapy (58). It is a major oncological problem reported in approximately 40% of patients undergoing standard dose chemotherapy and in almost all patients receiving high dose chemotherapy and stem cell transplantation (59). The prevalence of mucositis also varies depending on the type of cancer and therefore the combination of cytotoxic drugs (60). Patients with mucositis exhibit severe clinical symptoms including pain due to ulceration of the GIT, nausea, vomiting, heartburn, diarrhoea, constipation, and therefore severe weight loss. Furthermore, ulceration of the GIT is commonly associated with a high risk of systemic infection which poses a threat to patient health (61).

Mucositis can result in unplanned treatment interruptions including dosage reduction or premature cessation of cancer treatment. Patients may require prolonged hospitalization and administration of antibiotic, antiviral therapy, or antifungal drugs depending on the severity of the condition (60-61). Many treatment options are available to prevent and treat this condition, but none of them can completely prevent or treat mucositis. Currently, management of mucositis is largely supported with treatment limited to pain relief, maintenance of good oral hygiene, and the use of loperamide (a non analgesic opioid) to treat diarrhea (62). Thus, mucositis is a major clinical and economic burden that severely impacts patients' quality of life and increases their risk of morbidity and mortality (63).

3.5.1 Pathobiology of Mucositis

The pathobiology of mucositis is complex and involves the interplay of multiple intricate pathways including molecular and cellular events that occur in all layers of the gastrointestinal mucosa. Historically, it was assumed that mucositis development was simply an epithelial phenomenon and occurred due to the direct toxic effect of cytotoxic chemotherapeutic agents on the basal cells of the gastrointestinal tract epithelium (58). However, recent investigations have clearly identified that mucositis development is complicated with the involvement of molecular pathways between all mucosal compartments (64). Initially, mucositis development was proposed in a four-stage model by Sonis in 1998 (65). However subsequent studies have further extending this model into a five stage model comprising of the (1) initiation, (2) upregulation and message generation, (3) signalling and amplification, (4) ulceration and inflammation, and (5) healing phase (3, 65). (Figure: 3.6 and 3.7)

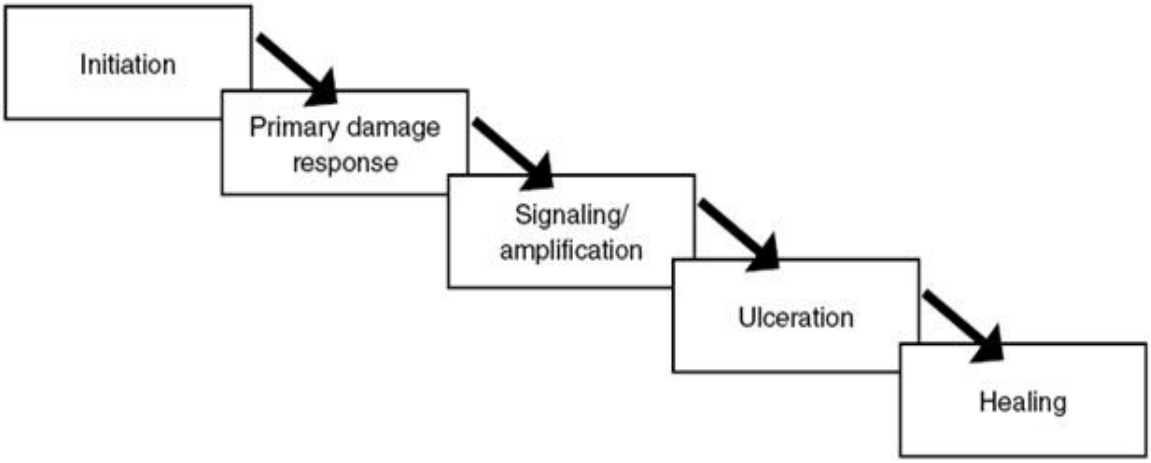


Figure: 3.6 Five stage model of mucositis (65)

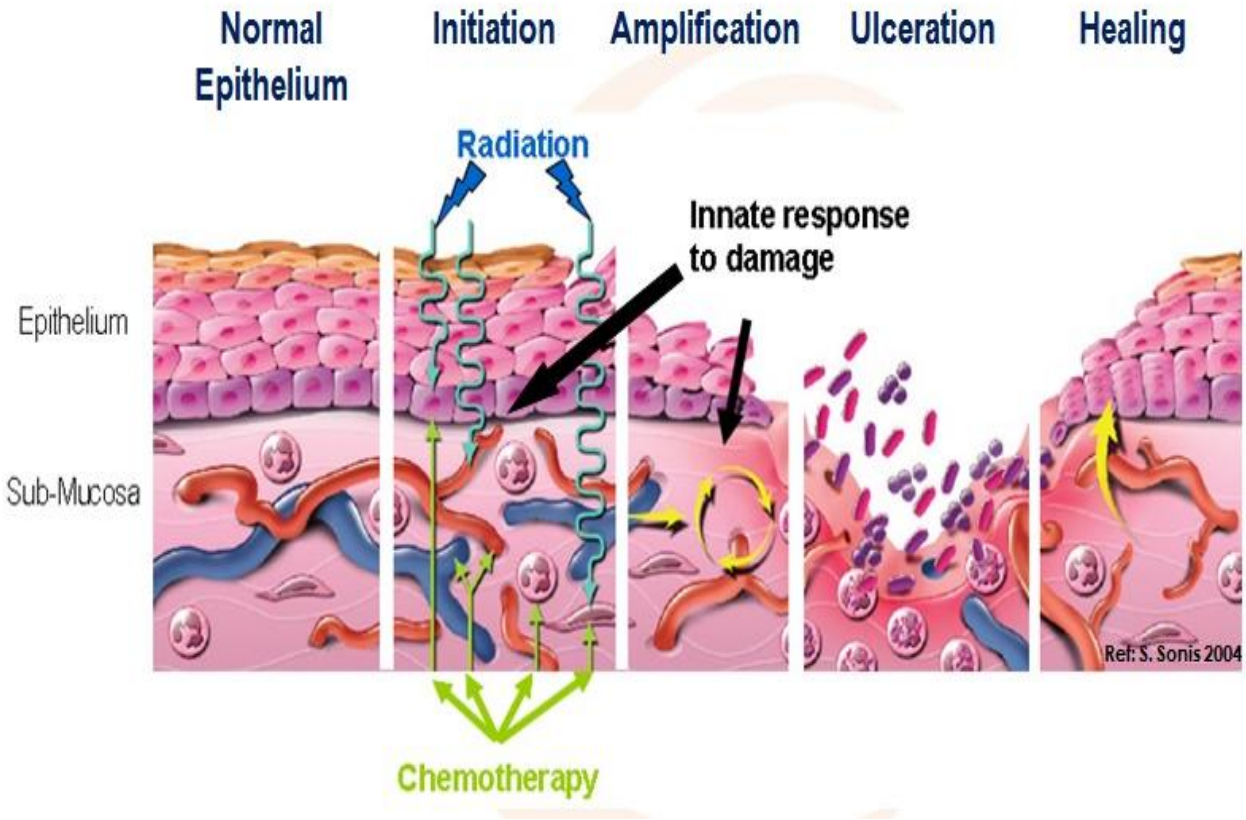


Figure: 3.7 - Biological phases of mucositis (65)

Briefly, **the initiation phase (Phase I)** occurs immediately following exposure to cytotoxic therapy resulting in direct damage to cellular DNA leading to immediate cell injury or death in the basal epithelia and submucosal cells (60). Furthermore, extensive tissue injury can result in the generation of reactive oxygen species (ROS). ROS are known to cause damage to cells and tissues, stimulate macrophages, and trigger a cascade of inflammatory pathways including the SP1-related retinoblastoma control protein, p53, and the nuclear factorkappa-B (NF-κB) inflammatory pathway (66).

The upregulation and message generation phase (phase II) involves the activation of a number of signalling pathways and transcription factors, most importantly NFκB (67). NFκB in turn mediates gene expression and synthesis of various inflammatory molecules including proinflammatory cytokines such as tumour-necrosis factor (TNF), interleukin-6 (IL-6), interleukin-1β (IL-1β), adhesion molecules, and cyclooxygenase-2 (COX-2) from adjacent connective tissue (68). The initiation of this inflammatory cascade has been confirmed in the clinical setting where studies have demonstrated the presence of these inflammatory factors in peripheral blood samples of chemotherapy patients (68).

Phase III of mucositis is the **signal amplification phase** where the inflammation signal is further amplified as a consequence of proinflammatory cytokines such as TNF acting in a positive feedback loop to reinforce NFκB activation (67). The net effect of this amplified inflammatory positive feedback loop is enhanced production of proinflammatory cytokines (TNF, IL-6, and IL-1β) and further tissue damage as a result of increased apoptosis.

Phase IV is the **ulcerative phase** wherein gastrointestinal epithelium integrity is destroyed and mucositis is clinically evident. The GIT epithelium is characterised by the formation of pseudomembranes and ulcers (69). Bacterial colonisation at the site of ulceration further induces inflammation and activates the infiltration of macrophages and other inflammatory cells to the site of tissue injury (70).

P**hase V: the final healing phase** occurs with in approximately two weeks following cessation of treatment and is a spontaneous self-resolving process where the intestinal epithelium is renewed. Although it remains the least understood stage of mucositis, it is thought that COX-2 may play an important "rebuilding" role in the healing phase by initiating angiogenesis which is predominantly seen in this phase. (3) (Figure: 3.8)

3.6 FREE RADICALS

Free radicals are highy unstable and reactive molecules that have unpaired electrons.They may formed from oxygen and/or nitrogen. The reactive oxygen species include superoxide anion, hydroxyl free radical (•OH), hydrogen peroxide etc. The superoxide free radical (O_2-•) formed by NADPH oxidase system and abundant production in mitochondria. The hydrogen peroxide (H_2O_2) is produced by superoxide dismutase and further form hydroxyl free radical (•OH) via fenton reaction and finally hydroperoxide radical (HO_2-•). The reactive nitrogen species includes free radical nitric oxide (NO•) synthesized from L-arginine by the action of nitric oxide synthase enzyme, the peroxynitrite (ONOO-) formed by the reaction of nitric oxide free radical with superoxide free radical which produce nitrogen dioxide (NO_2•) as an intermediate and finally generates anhydrous nitrous (N_2O_3) (71-72) (Figure: 3.9)

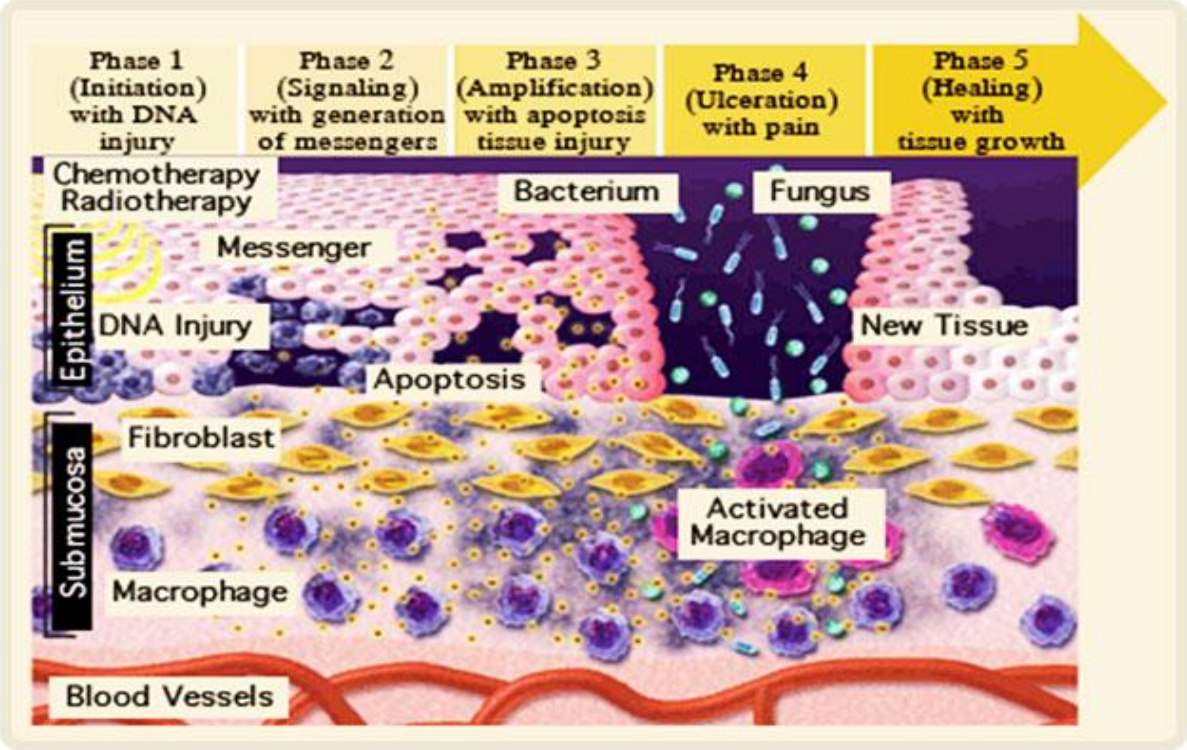


Figure: 3.8 – Phases of mucositis in response to chemotherapy/radiotherapy (3)

REACTIVE OXYGEN SPECIES		REACTIVE NITROGEN SPECIES	
Superoxide anion	$O_2 \xrightarrow[\text{oxidase}]{\text{NADPH}} O_2^{-}\bullet$	Nitric oxide	$\text{L-Arginina} \xrightarrow[\text{NOS}]{\text{L-Citrulina}} NO\bullet$
Hydrogen peroxide	$O_2^{-}\bullet \xrightarrow[\text{SOD}]{O_2^{-}\bullet,\ O_2} H_2O_2$	Peroxynitrite	$NO\bullet \xrightarrow{O_2^{-}\bullet,\ O_2} ONOO^{-}$
Hydroxyl radical	$H_2O_2 \xrightarrow{\text{Fenton reaction}} \bullet OH$	Dioxide of nitrogen	$NO\bullet_2$
Hydroperoxyl radical	$O_2 \xrightarrow{H\bullet} HO_2^{-}\bullet$	Anhydride nitrous	$NO\bullet \rightarrow N_2O_3$

Figure: 3.9 - Representative diagram of different reactive oxygen species and reactive nitrogen species (72)

3.7 REACTIVE OXYGEN SPECIES

3.7.1 Sources:

Dioxygen (O_2) is an essential molecule for the survival of the organism which is produced by the mitochondrial respiration process. The partially reduced metabolites of the oxygen from mitochondria such as superoxide anion, hydrogen peroxide, hydroxyl radical, which are highly reactive are termed as reactive oxygen species (ROS) (73-74). (Figure: 3.10)

3.7.2 Defense against ROS:

ROS is considered as toxic by-product of oxygen metabolism and cellular response due to hazardous stimuli cause damage to carbohydrates, lipid, proteins and DNA. The superoxide anion production is mediated by NADPH oxidase and xanthine oxidase. The superoxide mediated toxicity is moderated by cellular defense mechanisms by antioxidants superoxide dismutase, catalase, vitamin E, thiol peroxidases, glutathione-S-transferase, glutathione peroxidase and reduced glutathione (75) (Figure: 3.11).

3.7.3 Oxidative stress and its markers:

Oxidative stress is caused due to an imbalance between ROS production and its detoxification by the antioxidant defense system. ROS induced peroxidation of membrane lipids cause changes in biological properties, degree of fluidity, inactivation of membrane receptors leading to increase in tissue permeability and impair the normal cellular function. This lipid peroxidation is measured by the formation of its end products malondialdehyde, 2-propenal, 4-hydroxy-2-nonenal and isoprostanes. Proteins are the major targets for ROS. This alters the structure of protein leading to cross linking, peptide cleavage, modification of side chain aminoacid and

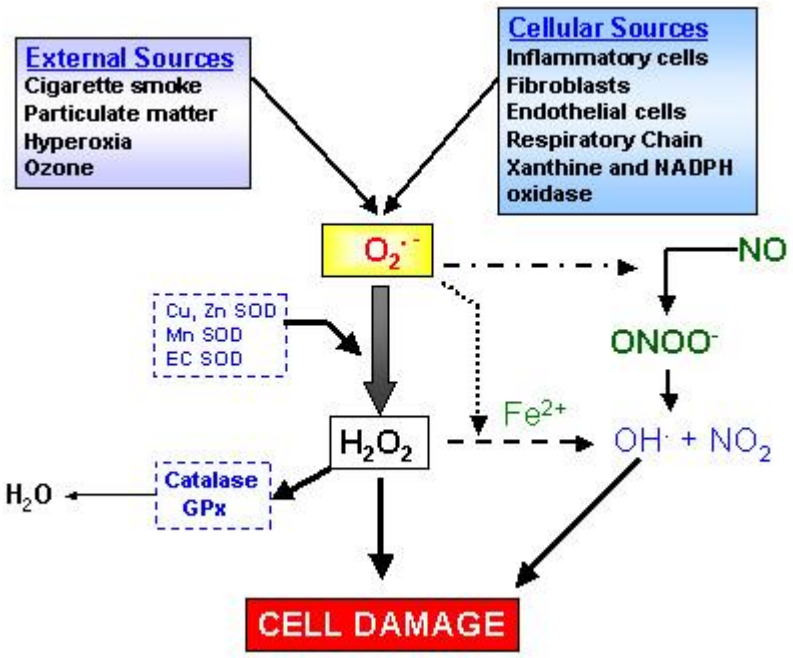


Figure: 3.10 - Formation of reactive oxygen species and it effect (74)

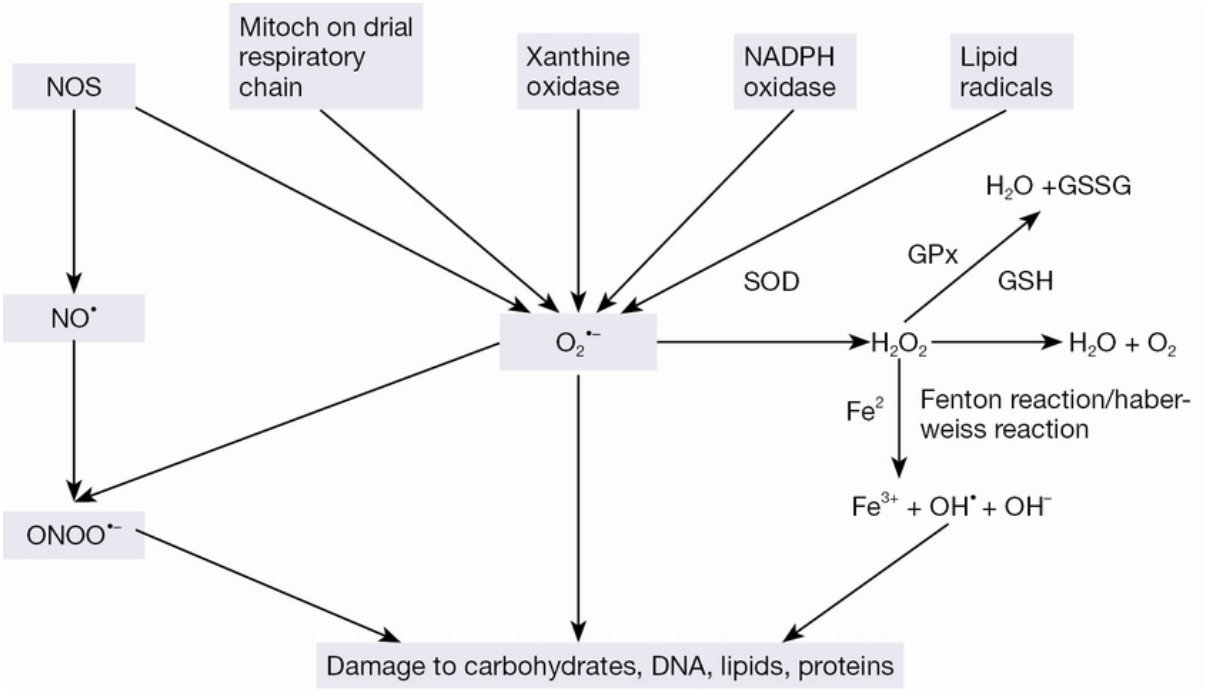


Figure: 3.11 – Effect of ROS on biomolecules (75)

changes in the functional properties. Measurement of protein carbonyl acts as a marker for the oxidation of side chain aminoacids. ROS also damages the DNA molecule by hydroxyl group causing loss in fidelity, inactivation of DNA repairing enzymes and generates sugar, base modification products. 8-hydroxy-20-deoxy guanosine was measured as index of DNA damage due to oxidative stress (76).

3.7.4 ROS and small intestines

The intestinal tract is continually attacked by luminal microbes and by oxidized compounds from the diet, exposing it to recurrent oxidative changes. Intestinal epithelial cells act as a selective permeable barrier, which allows the absorption of nutrients, electrolytes and water by transcellular and paracellular pathways, also affording their intracellular compartmentalization and trafficking towards the body. These cells are able to regulate the traffic of antigens towards gut-associated lymphoid tissues, in order to discriminate between innocuous and pathogenic antigens, acting as a crossroad between tolerance and the immune response.

ROS and their oxidized products may be considered as part of a network signaling system controlled by antioxidant defenses. For instance, moderate quantities of ROS can act as biological signal molecules, which are involved in different phases of the inflammatory immune response and of autophagic processes activated by luminal agents in intestinal cells. These events imply the production of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{-\bullet}$) or nitric oxide (NO) at specific intracellular sites, i.e. mitochondria, membrane nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase, endothelial inducible NO synthase (iNOS) and myeloperoxidase in inflammatory cells (77). H_2O_2 regulate redox sensitive transduction

pathways, such as phosphatidylinositol 3-kinase/AKT, mitogen-activated protein kinase/extracellular signal regulated kinase kinase / extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase, and also regulate activation of the oxidative stress-responsive nuclear transcription factor-κB (NF-κB), which is involved in inflammatory reactions (78). However, although cell inflammation and oxidative reactions are considered to be a primary host defense, excessive inflammatory reactions, with overproduction of $O_2^{-\bullet}$, H_2O_2, NO and HOCl by activated leukocytes, can overwhelm the tissue′s antioxidant defenses and may contribute to functional impairment of the enteric mucosa, leading to an aberrant response to luminal agents. These events have been extensively considered in the pathogenesis of inflammatory Bowel disease (IBD), drug, radiation, chemotherapy, toxin induced chronic abnormal inflammatory and immune responses (79).

An antioxidant intestinal environment reflects the intestinal mucosa′s response aimed at preventing oxidative damage, and is maintained by a complex dynamic recycling system in which different molecules undergo well-established oxido-reductions. The chief molecules involved are antioxidant enzymes, i.e. superoxide dismutase, catalase and glutathione peroxidases, as well as non-enzymatic molecules some of which originate from the diet, such as ascorbic acid, tocopherols and amino-thiols compounds (80) (81). (Figure: 3.12)

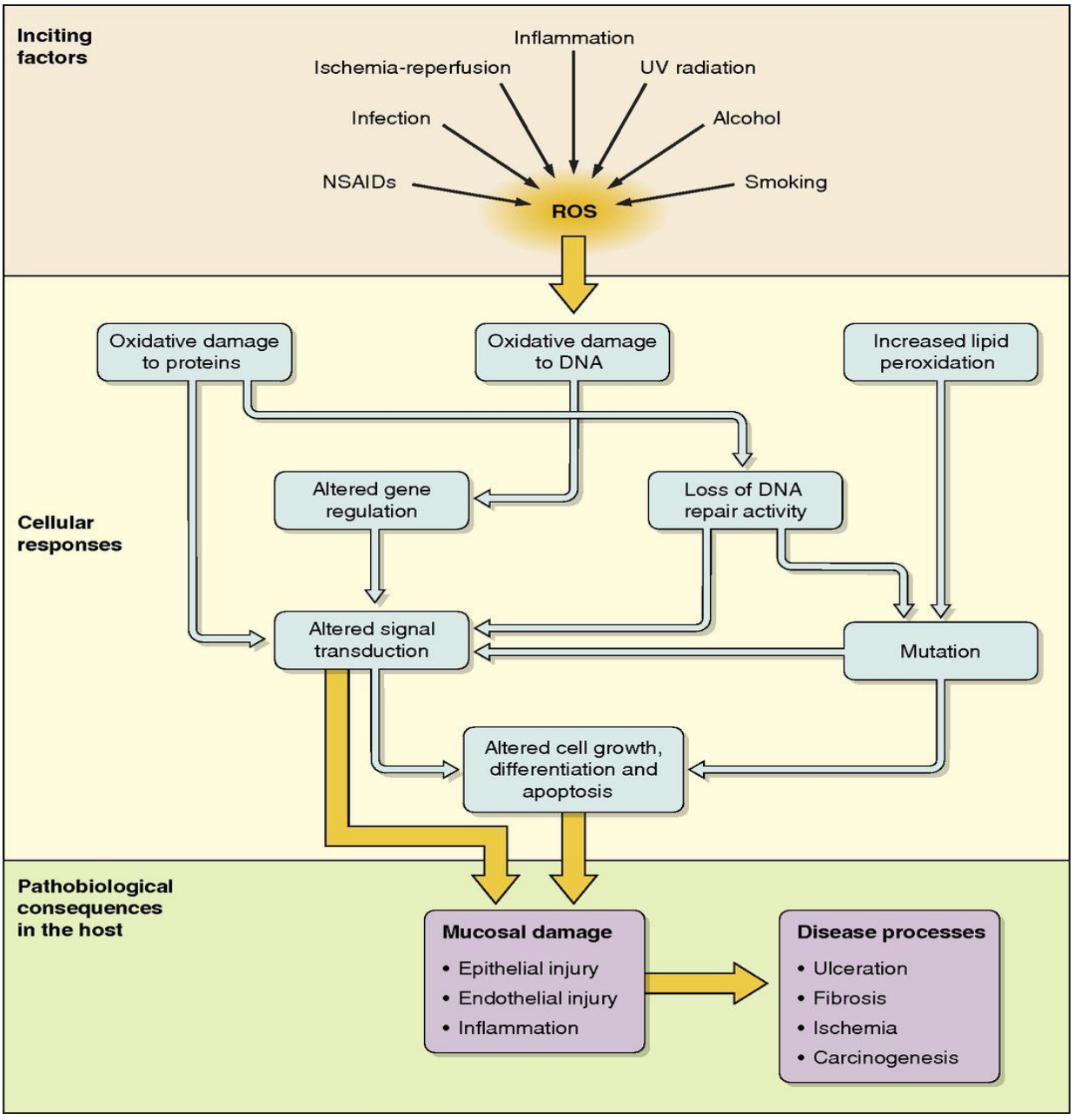


Figure: 3.12 – Cellular response and pathobiological consequence of ROS in intestine (81)

3.8 REACTIVE NITROGEN SPECIES

3.8.1 NITRIC OXIDE:

Nitric oxide was first identified and synthesized in mammalian cells by Louis Ignarro in 1986 (82). It is an inorganic gas soluble at 2mM concentration in aqueous solutions. It is highly reactive short lived molecule which has half life of few seconds and produced by a group of nitric oxide synthase (NOS) enzymes. NO play role in biological processes like immune defense, cell motility and cell death. Nitric oxide is synthesized from L-arginine by nitric oxide synthase enzyme. The biosynthesis of NO inside the cells is regulated by changes in NOS expression, distribution of NOS within cells, enzymatic activation of NOS and cellular inhibitors of NOS activity. (83) (Figure: 3.13)

Nitric oxide has diverse physiological functions like vasodilation, neuronal and immune function, inflammation and regulation of apoptosis. The effect of NO in the cellular functions depends on its concentration. During pathological conditions, the NO concentration seems to be higher within the cell. Nitric oxide induces inflammation by the activation of the proteins of inflammatory mechanism such as TNF-α, NF-κB, COX2 etc. Nitric oxide has the ability to induce or to protect cells from apoptosis in different cell types based on its dose within the cells. The apoptotic or anti-apoptotic effect of nitric oxide depends on the cellular protein functions like release of cytochrome c from mitochondria, activation of caspase cascade system, expression of p53, hsp70, Bcl-2 and Bcl-XL as well as cGMP signaling (84). (Figure: 3.14)

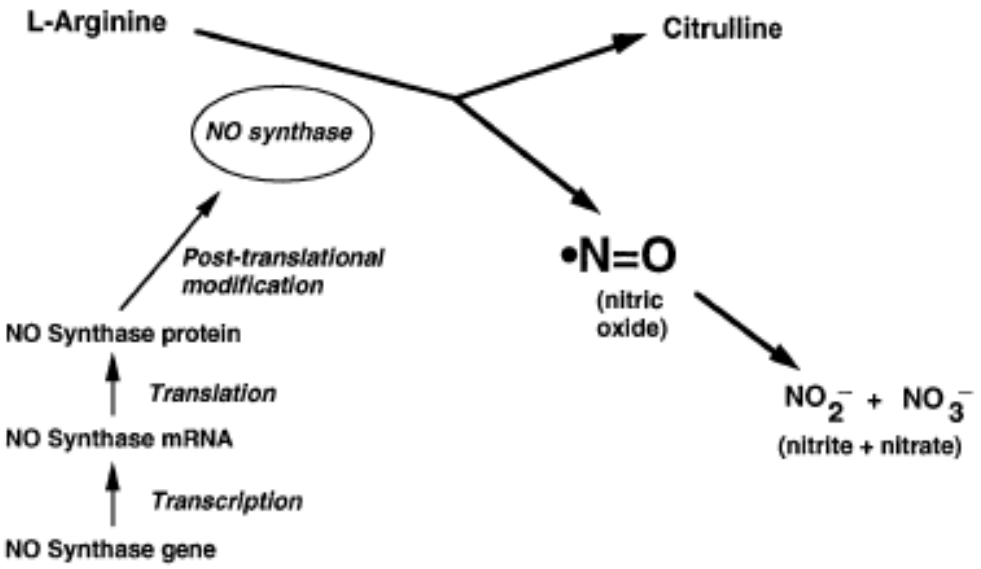


Figure: 3.13 - Biosynthetic pathway of nitric oxide (83)

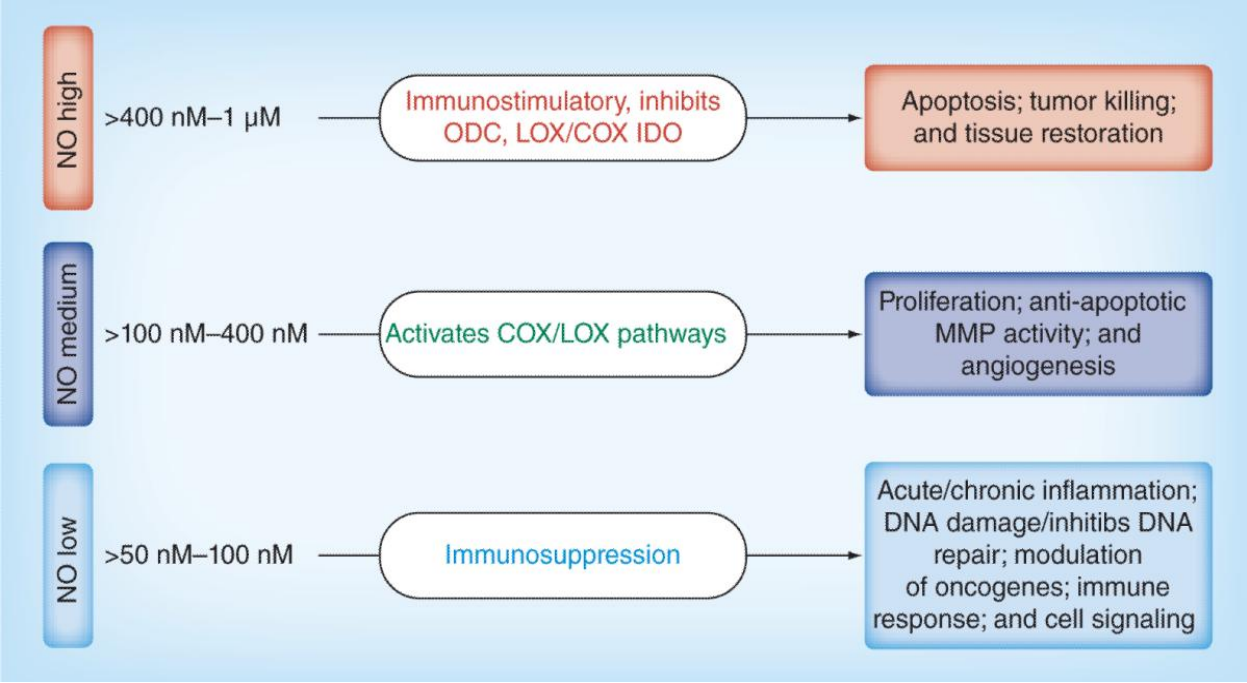


Figure: 3.14 - Pleiotropic effect of nitric oxide on inflammation and apoptosis (84)

3.8.2 Sources of NO

Nitric oxide (NO) is synthesized by nitric oxide synthase (NOS). NO can also be produced non enzymatically by reaction of hydrogen peroxide and arginine, by xanthine oxidase or by reduction on nitrites in acidic medium (85). NO has half life of 3-5s quickly react with superoxide anion and forms peroxynitrite which has an half life of 1-2s (86) under physiological conditions. NO converted into reactive nitrogen species such as nitroxyl anion, nitrosonium cation or peroxynitrite depending on the environment. Peroxynitrite is a major cytotoxic agent produced during sepsis, ischemia/reperfusion and inflammation. (87) (Figure: 3.15)

3.9 NITRIC OXIDE SYNTHASES:

Nitric oxide synthases belongs to a family of cytochrom p450 complex like hemeproteins depends on oxygen, NADPH, flavin, tetrahydrobiopterin and catalyze the oxidation of L-arginine to form nitric oxide (88). There are four types of NOS namely endothelial NOS (eNOS), neuronal NOS (nNOS), inducible NOS (iNOS) and mitochondrial NOS (mtNOS) (89). All these type found in most all the tissues as well as seen in same tissue. Nitric oxide synthases have wide physiological functions but their overactivation can have harmful effects especially on the proteins (90). Among NOS types, eNOS and nNOS are constitutively expressed in tissues, whereas iNOS is an inducible form expressed during the inflammatory and immunological responses. eNOS, nNOS and iNOS are found in the chromosomes 7, 12, and 17 respectively (88). The nNOS has a molecular weight of 160 or 140 kDa where as iNOS and eNOS have approximately 130kDa. Endothelial NOS and neuronal NOS produce low level of nitric oxide controlled by calcium calmodulin system at physiological condition. Important functions of eNOS are vasodilation, vasoprotection, immune regulation, antiproliferative and antioxidative

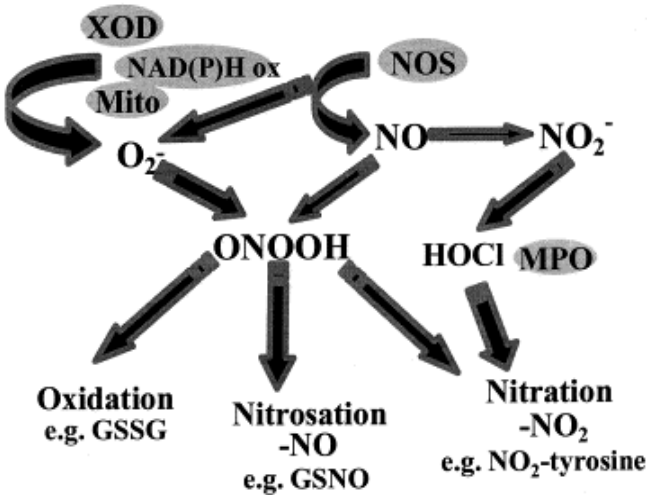


Figure: 3.15 - Formation of reactive nitrogen species depending nitric oxide and its effect (87)

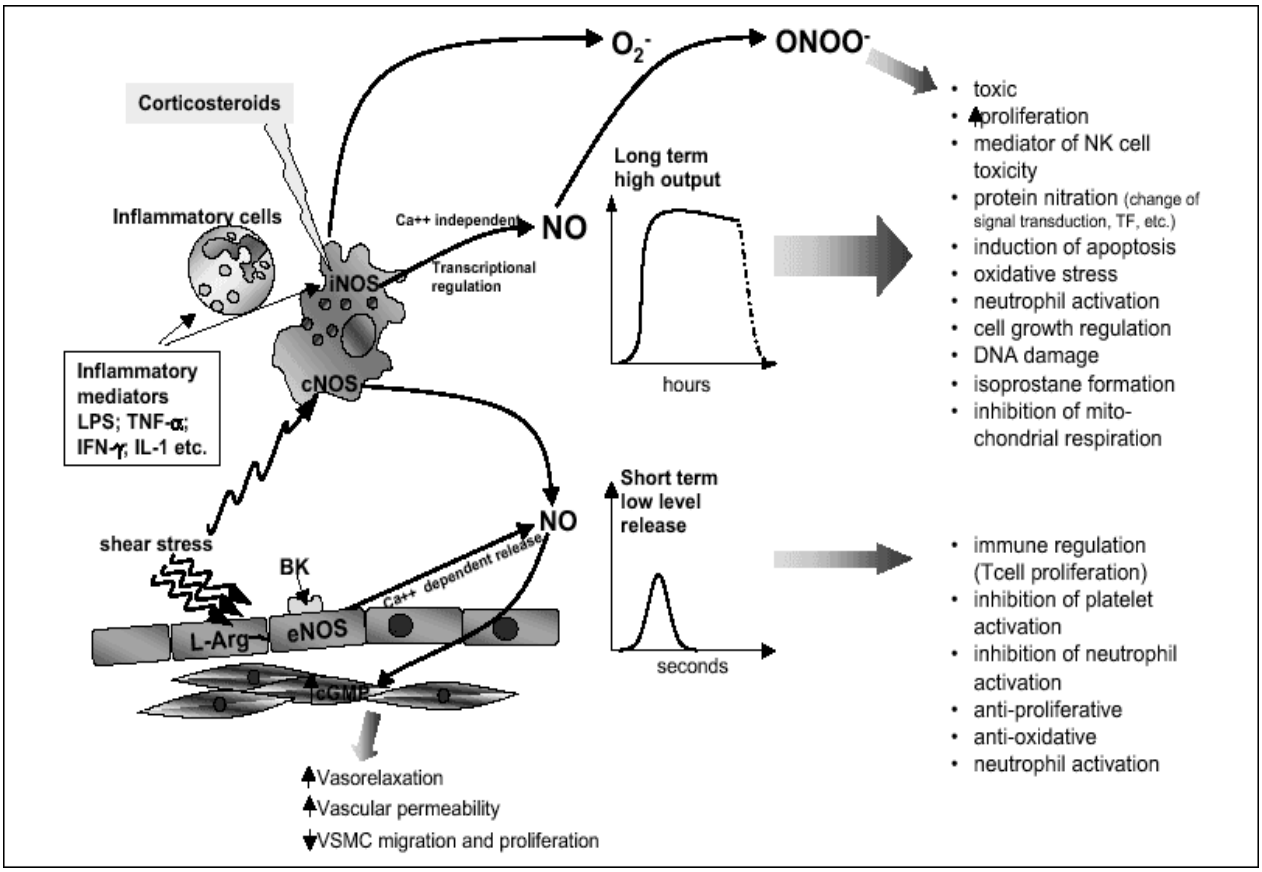


Figure: 3.16 – Biological and pathological effect of NOS (91)

effects. nNOS is involved in cell communication like synaptic plasticity, blood pressure regulation, penile erection, and neurotransmission. iNOS is produces high levels of nitric oxide and is involved in non specific immune defense, septic shock, neutrophil infiltration, oxidative stress, DNA damage, and mediation of inflammation, protein nitration, inhibition of mitochondrial respiration and induction of apoptosis (91). (Figure: 3.16)

3.10 INDUCIBLE NITRIC OXIDE SYNTHASE:

Inducible nitric oxide synthase (iNOS), has been reported to be expressed in large variety of cells including macrophages, monocytes, neutrophils, eosinophils, liver and kidney cells of rats, muscle cells, epithelial cells, neuronal and endothelial cells (82). iNOS derived nitric oxide plays numerous important physiological functions such as wound repair, regulation of blood pressure and defense mechanism in host cells. It is induced by pathological stimuli such as increase in tumour necrosis factor alpha, lipopolysaccharides, cytokines and endotoxins (92).

During inflammatory responses, iNOS produces large amounts of nitric oxide which when sustained for long time causes cytotoxic effect. iNOS, is transcriptionally regulated by nuclear factor κB (NF-κB) and interferon regulator factor-1 (IRF-1). NF-κB is bound to its inhibitor IκB and is present in the cytoplasm. During inflammatory stimuli IκB gets phosphorylated and NF-κB translocates into the nucleus and triggers iNOS transcription (93). This process is inhibited by preventing IκB degradation. Other signaling pathways that stimulate iNOS expression are tyrosine kinase, janus kinase, protein kinase C, mitogen activated protein kinase and raf-1 protein kinase (94).

Lipopolysaccharides, cytokines like TNF-α, interleukin -1b and interferon gamma binds to its respective receptors and activates the NF-κB, IRF-1 and STAT-1 pathway. This leads to the transcription of iNOS gene and thus increases the iNOS expression in the effector cell. Over expression of iNOS in the cell results in the production of increased level of nitric oxide which has its adverse effect on the target cell. (95) (Figure: 3.17)

Nitrosative stress:

Nitrosative stress is defined as cellular stress in which reactive nitrogen species acts along with the reactive oxygen species. The reactive nitrogen species including nitric oxide ($NO^{\bullet}$), nitrosyl cation [NO^{+}], nitroxyl anion [NO^{-}], peroxynitrite [$ONOO^{-}$], nitrogen dioxide ($NO_2^{\bullet}$), dinitrogen trioxide [N_2O_3], dinitrogen tetroxide [N_2O_4], nitrosothiols [RSNO] are responsible to induce and to detect nitrosative stress.

Defense against nitrosative stress:

Enzymes like nitric oxide reductase, denitrase, denitrosylase and S-nitrosoglutathione reductase protects cells from nitrosative stress (96) also nitrosothiol and nitrosoglutathione.

Nitrosative stress markers:

The major target of RNS was proteins leading to changes in the aminoacid moiety and cause alterations in the biological function of proteins. Nitric oxide mediated post translational protein modification include binding to metal centers, oxidation of thiols (cysteine and methionine), nitration (tyrosine, tryptophan, carboxylic acid, amine, phenylalanine group), and nitrosylation (thiol and amine groups). However much attention is paid to protein tyrosine nitration (nitration

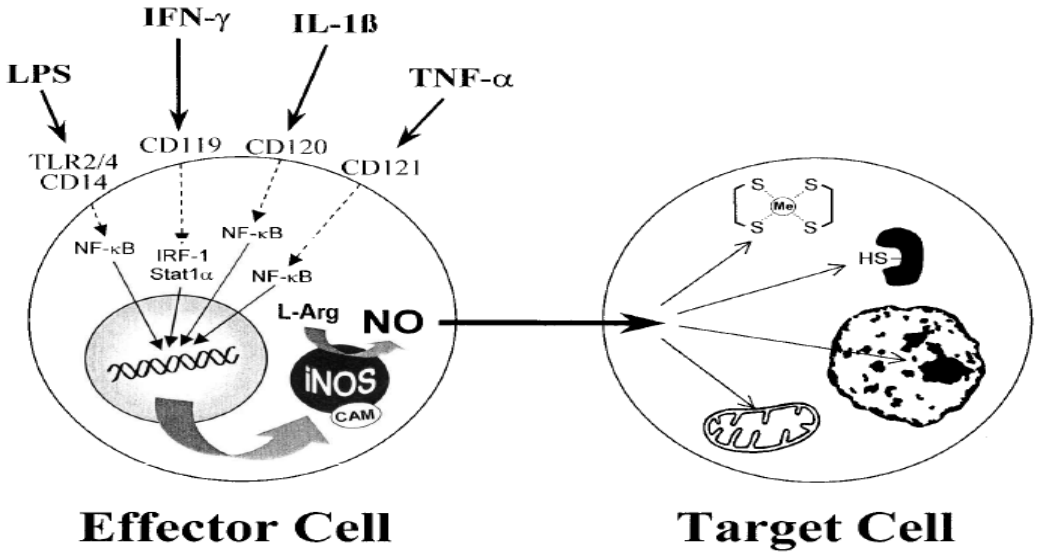
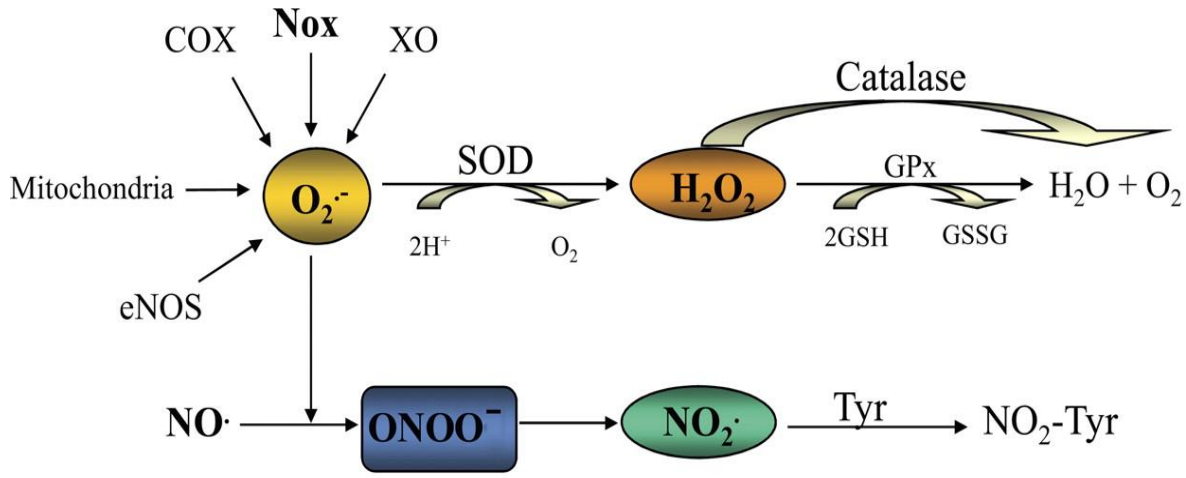


Figure: 3.17 - iNOS induction pathways and its cellular targets (95)



XO: xanthine oxidase
Nox: NADPH oxidase
$O_2^{\cdot-}$: superoxide anion
SOD: superoxide dismutase
H_2O_2: hydrogen peroxide
GPx: gluthatione peroxidase
GSH: reduced gluthatione
GSSG: oxidized gluthatione
NO·: nitric oxide
$ONOO^-$: peroxynitrite
NO_2· : nitrogen dioxide
NO_2-Tyr: nitrotyrosine

Figure: 3.18 – Peroxynitrite production and tyrosine nitration (98)

of tyrosine residue) and protein S nitrosylation (nitrosylation of thiol) which may have a crucial role in nitric oxide mediated cellular signaling mechanisms (97) (98) (Figure: 3.18). Analysis of 3-nitrotyrosine is a prominent marker for measuring nitric oxide derived oxidant. 3-chlorotyrosine, 3-bromotyrosine and dityrosine also measured in various disease conditions as marker (76). Protein tyrosine nitration and protein cystein nitrosylation can be analysed by various methods such as western blot, immunoprecipitation, immunofluorescence, two dimentional electrophoresis followed by mass spectrometry (97).

NO and small intestines

Nitric oxide (NO) plays a critical role in several physiological processes that occur in the GI tract including motility, secretion, digestion, absorption and elimination. The importance of NO in GI mucosal defense is well established (99). NO contributes to mucosal defense through its cytotoxic properties, a primary defense against ingested bacteria and parasites (100). The actions of NO overlap considerably with those of prostaglandins: modulation of the activity of mucosal immunocytes (e.g., mast cells and macrophages), reduction of leukocyte-endothelial adhesive interactions, modulation of mucosal blood flow, reduction of epithelial permeability, stimulation of mucus, and bicarbonate secretion. NO has proven to be the primary non-adrenergic non-cholinergic neurotransmitter in the GI tract (101).

The GIT expresses three types of NOSs. The constitutive (calcium dependent) isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS), produce small amounts of NO which acts as a neurotransmittor and vasodilator respectively. The inducible (calcium independent) isoform (iNOS) produces much larger amounts of NO and is expressed during inflammation. iNOS is

induced by cytokines like interferon-gamma (IFN-γ), tumor necrosis factor α, interleukin-1 (IL-1) and lipopolysaccharide (LPS). iNOS induction is suppressed by transforming growth factor (TGF-β), and interleukin -8 and -10 (IL-8,-10). Recent studies indicate that iNOS is expressed in a small amount and plays a role in intestinal mucosal integrity under physiological conditions (102). There is a general agreement that NO derived from the constitutively expressed NOS (e.g., eNOS or nNOS) is protective/anti-inflammatory and the large amounts of NO derived from the inducible iNOS is proinflammatory. High concentrations of NO are related to numerous pathological processes of GIT including peptic ulcer, chronic gastritis, gastrointestinal cancer, bacterial gastroenteritis, celiac or chronic inflammatory bowel diseases (103)

3.11 PHYSIOLOGICAL AND PATHOLOGICAL ROLES OF ROS/RNS:

ROS and RNS, at higher concentrations cause damage to all cellular constituents. At low or moderate concentration play a regulatory role in cellular signaling processes. The physiological functions include vascular tone, erythropoietin production, monitoring oxygen tension, control of signal transduction (104) defense against infectious agents, induction of mitogenic response, and maintenance of redox homeostasis where as excessive production leads to consequences to its downstream causing tissue injury (71). It is also shown that these species have a role in necrosis or apoptosis, induction or suppression of many genes, and activation of cell signaling cascades (105). ROS / RNS induced stress has been implicated in pathogenesis of disorders including cancer, atheroscelerosis, chronic inflammation, hypertension, neurodegenerative diseases, rheumatoid arthritis, cardiovascular diseases, HIV infection, aging, diabetes, ischemia-perfusion injury (104) (71) (106) (Figure: 3.19)

Physiology — Consequence/function: — Pathophysiology

ROS/RNS involvement in physiological processes	ROS/RNS involvement in pathophysiological processes
Regulation of vascular tone	Age related diseases (aging)
Regulation of cell adhesion	Malignant transformation
Amplification of immune response	Atherosclerosis
Programmed cells death	Neurodegenerative disease
Receptor mediated signaling pathways	Rheumatoid arthritis
	Ischemia reperfusion injury
	Obstructive sleep apnea
	Obesity and diabetes

Figure: 3.19 – ROS/RNS physiological and pathological process (106)

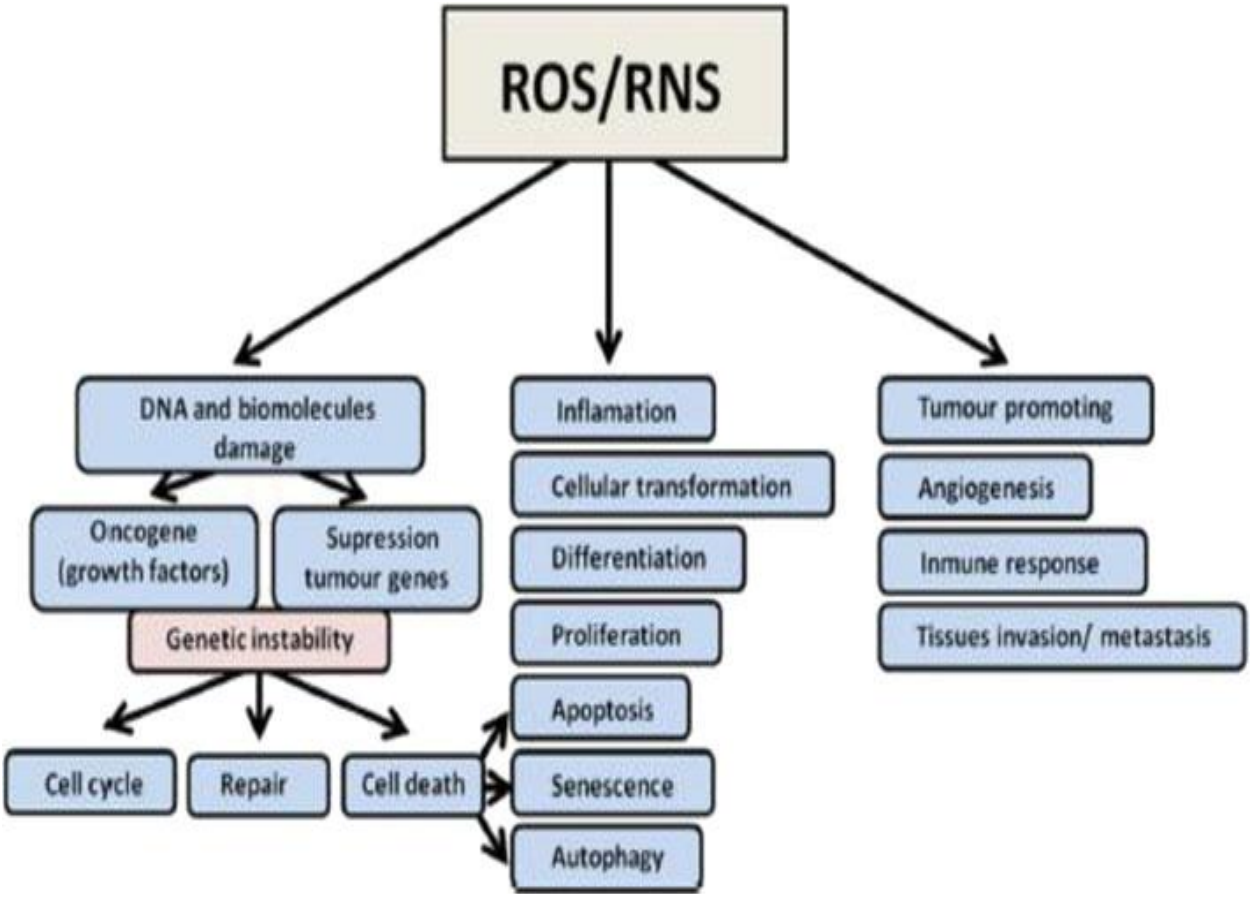


Figure: 3.20 - Role of ROS/RNS in carcinogenesis process (72).

Reactive oxygen species and reactive nitrogen species produce damage to biomolecule and DNA causing genetic instability and affecting the mechanism of cell cycle, cell repair and cell death. Free radicals are essential for cellular transformation, differentiation and proliferation and evalulation of inflammation process. It has been implicated that these free radicals are involved in the carginogenesis by promoting tumour progression, invasion and metastasis (72) (Figure: 3.20)

3.12 PEROXYNITRITE:

Nitric oxide and superoxide are the precursors of peroxynitrite. Nitric oxide produced by nitric oxide synthase reacts with superoxide generated by NADPH oxidase, mitochondrial respiratory chain, respiratory burst, xanthine oxidase and autooxidation of biomolecules to form peroxynitrite which is responsible for nitrosative stress leading to implications on several biomolecules and changes in biological process during inflammation. Peroxynitrite is a short lived free radical than its precursor but stable and long lived compared to peroxynitrous acid. The exact half life is not known but it is reported as 10 millisec in extracellular components. Under physiological conditions it has an half life of 1-2s. PON is a potent oxidizing agent. The cytotoxic effects of PON at the cellular level in the cell signaling pathways results in the modifications of targets which leads to various pathological consequences in diseases. The biological targets of peroxynitrite cytotoxicity are NFκB activation, protein modification and alteration in cell signaling (107). It also acts through mitochondrial components and causes alteration in membrane permeability and lowered mitochondrial energy production (108). (Figure: 3.21)

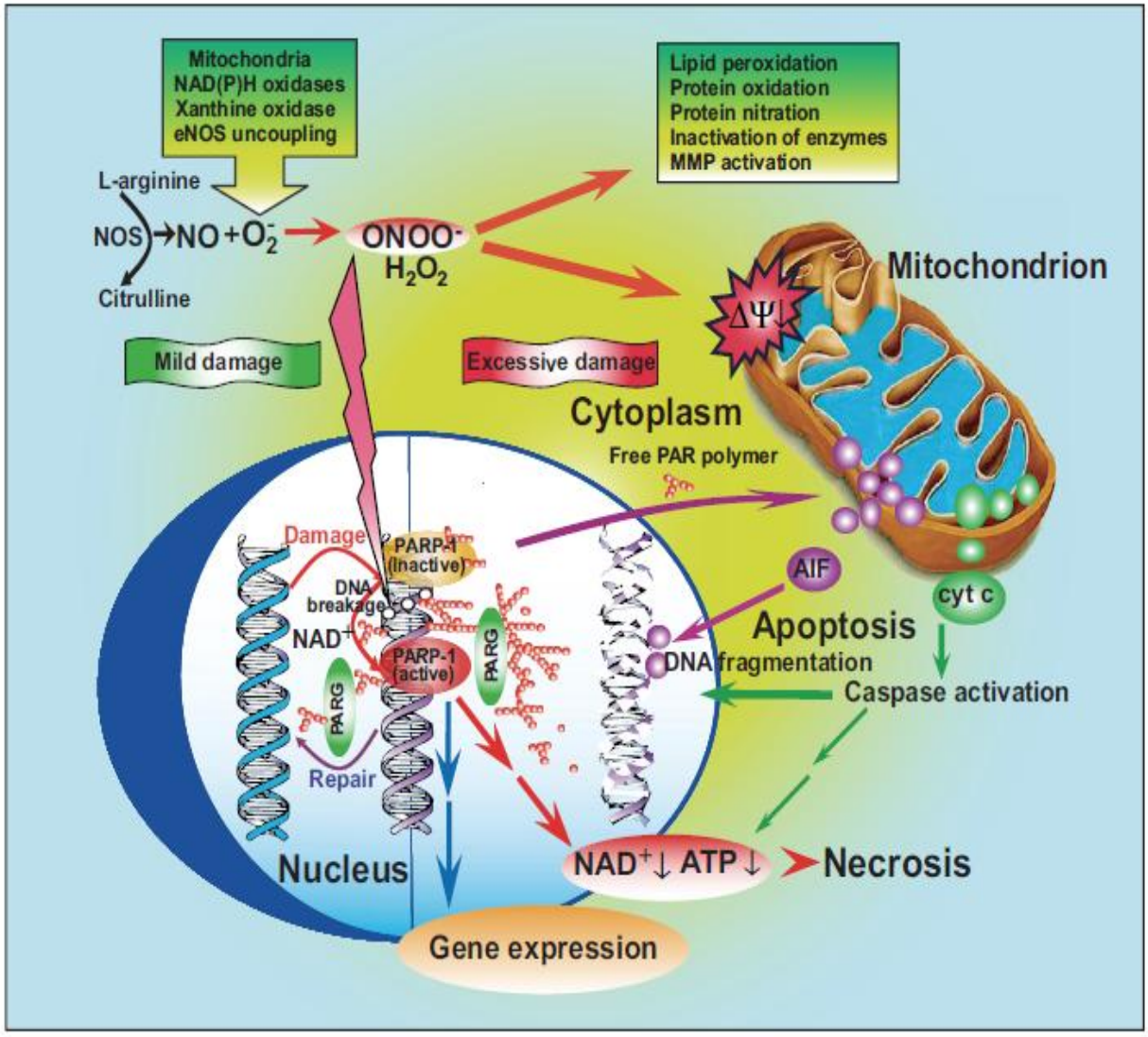


Figure: 3.21 - Mechanism of peroxynitrite mediated cell death (106)

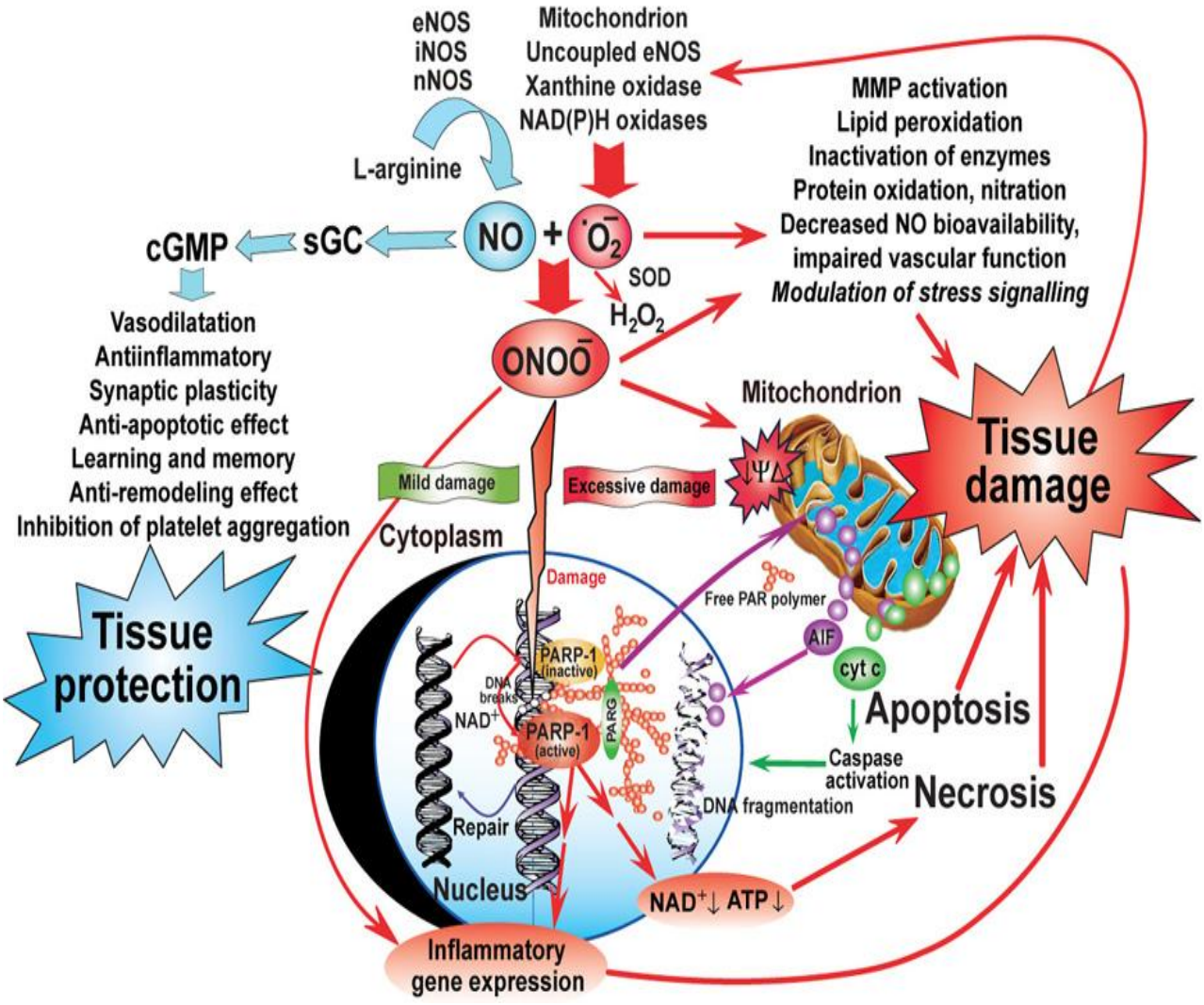


Figure: 3.22 – Biological and pathological effect of NO and ONOO on tissues (106)

Peroxynitrite is a major molecule which favours nitrosative stress by causing modifications in proteins by tyrosine nitration and cystein nitrosylation (98). The biochemical and pathophysiological effects of peroxynitrite include inhibition of antioxidant enzymes (SOD and glutathione reductase), inhibition of cytosolic enzymes (aconitase, creatine kinase and glyceraldehydes e phosphate dehydrogenase), aggregation of proteins, activation of enzymes (MMPs, cytochrome c , protein kinase C, fibrinogen), impairment of cofactors, inhibition of membrane channels and modification of cell signaling mediators leading to consequences like deleterious effect, causing injury, impairment in cell function, ionic imbalance and activation/impairment of inflammation and apoptotic signaling (109). The modifications in the cellular mechanism due to the effect of nitrc oxide and peroxynitrite can result in necrosis and apoptosis (106). (Figure: 3.22)

3.13 PROTEIN TYROSINE NITRATION:

3.13.1 Introduction:

Protein tyrosine nitration is a post translational modification of protein that occurs due to the reaction of protein with nitrating agents. Protein nitration is the addition of nitro group on proteins mainly tyrosine residue forming 3-nitrotyrosine. Under inflammatory conditions nitration affects 1-5 tyr residues of every 10,000 tyr (110). The biological consequences of protein tyrosine nitration include alterations in the enzymatic activity, modifications in protein phosphorylation, proteolytic degration, immunogenicity and implication on diseases (111). Not all the proteins or not all the tyrosine residues in the proteins undergo nitration. The selectivity of tyrosine nitration in proteins depends on location of tyrosine residue, exposure of the aromatic ring and protein association with microenvironment (110). Tyrosine nitration modifies the

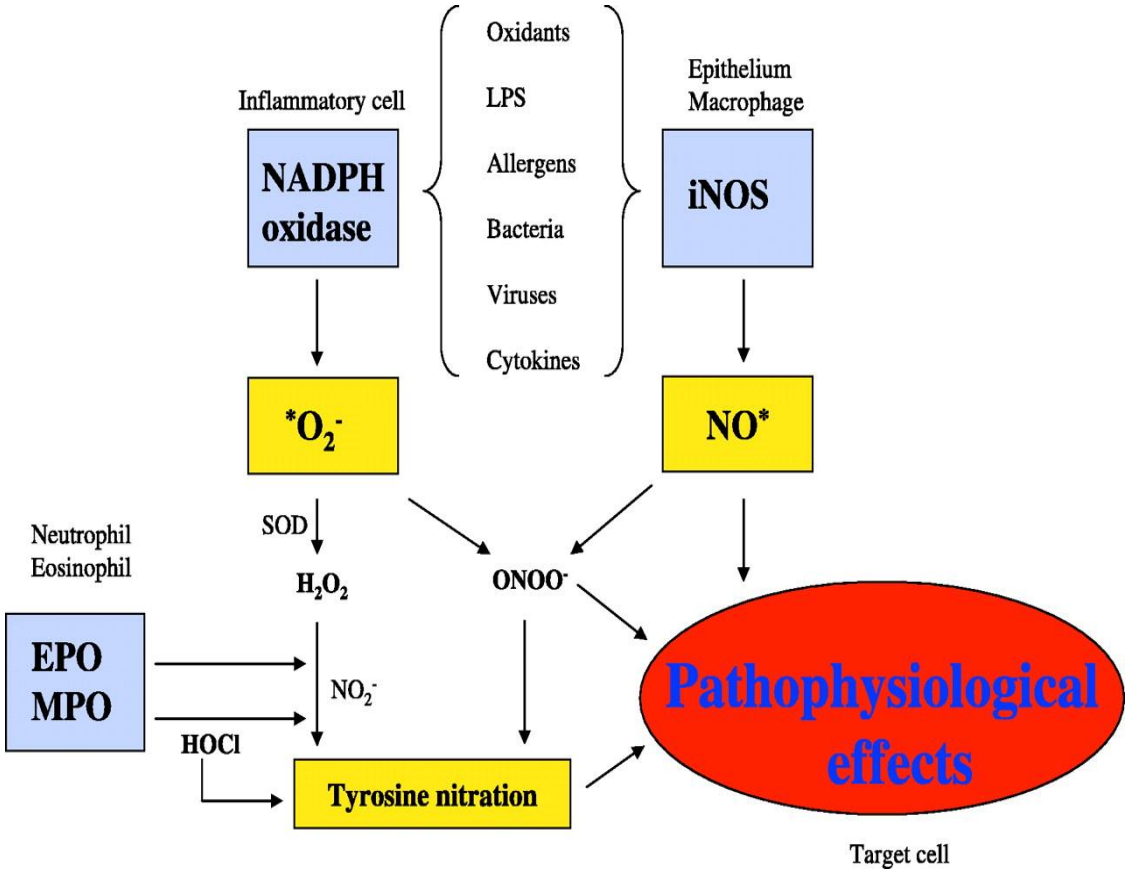


Figure: 3.23 – Mechanism of Protein tyrosine nitration (113)

properties of aminoacid like redox potential, pKa, volume and hydrophobicity. Protein nitration also affect regulation of cell cycle (112). Protein 3-nitrotyrosine is being used as a biomarker for nitrooxidative stress in cells and tissues (113) (Figure: 3.23).

3.13.2 Consequences of nitration:

Tyrosine nitration of proteins leads to gain or loss of function of proteins. Nitration cause conformational changes of proteins and reduces its activity leading to loss of function. The biological consequences of protein tyrosine nitration include enzyme activity modification, proteolytic degradation sensitivity, immunogenicity, protein phosphorylation alterations and implications in diseases. Nitration of NFκB/IκB triggers dissociation of IκB from NFκB and activates the pathway which is involved in cancer and inflammation (111). Examples for loss of function of some protein modification are Cu-Zn SOD, MnSOD, actin, glutamine synthase, inactivation of heme oxygenases, iron regulatory protein 1, decrease in histone deacetylase 2 increases inflammatory gene expression followed by histone acetylation, Aldolase, prostacyclin synthase (114), and gain of function of proteins are protein kinase C, fibrinogen, and cytochrome c. Cyt c nitration leads to decrease in mitochondrial respiration and stimulation of the apoptosis signaling (115), inactivation of glutathione S transferase by nitration causes marked reduction in lipid peroxidation inhibition which raise degree of membrane damage under pathological conditions (116), JNK nitration activates poly ADP ribose synthetase and leads to cell death (90). The nitrated proteins can be detected by using fluorescence imaging, histochemistry, western blot and proteomic based methods (111).

3.13.3 Nitration in health and disease:

Elevated levels of protein nitration is seen in wide range of disease conditions like stroke, chronic heart failure, diabetes, myocardial infarction, circulatory shock, cancer and neurodegenerative diseases (106) (109). Selected nitrated proteins have consequence in various diseases such as coagulation disorder, platelet dysfunction, sepsis, scelerosis, alzheimer's and parkinson's disease, inflammation and ischemia, endothelial dysfunction, cardiovascular and neurological disorders (117).

3.13.4 Peroxynitrite, tyrosine nitration and small intestines

Studies have shown that sustained NO production and ONOO- formation occurring in inflammatory states may differentially accelerate apoptosis in the villus apex and/or inhibit proliferation at the base of the crypts resulting in expanded extrusion zones at the villus tip resulting, at least transiently, in a "bare area" at the villus tip where bacteria can attach and traverse the epithelium. Peroxynitrite may promote gut barrier failure not only by inducing enterocyte apoptosis but also by disrupting signaling pathways involved in enterocyte proliferation (118-119).

3.14 PROTEIN- S - NITROSYLATION:

Protein S nitrosylation is a post translational modification of protein that occurs when nitric oxide reacts with cysteine thiol residue. Protein s nitrosylation play a key role in cell signaling, phosphorylation, ubiquitylation, acetylation of related protein modifications and alteration in cysteine based redox modifications. The specificity of S-nitrosylation depends on cellular redox environment, thiol microenvironment, activity of denitrosylases and transnitrosylases. As a

novel mechanism under physiological conditions, nitric oxide directly reacts with thiols in presence of electron acceptor forms s-nitrosothiol. The transfer of nitric oxide to the cysteine sulfhydryls on many proteins under *in vivo* condition results in the formation of S-nitrosothiols. These nitrosylated proteins have role on processes like signal transduction, host defense, DNA repair, blood pressure control and neurotransmission (120).

3.14.1 Consequences of nitrosylation:

The biological targets of nitrosylation are G proteins (p21, cdc42, rac1), enzymes (caspases, GAPDH, aromatases, transglutaminases) and kinases (JNK, ERK, p38). The NMDA receptor activity is downregulated by S-nitrosylation and causes redox modulation and decreases in channel opening under physiological condition. Glyceraldehyde -3- phosphate dehydrogenase activity is modulated by s-nitrosylation which promotes its apoptotic effect. Bcl2 nitrosylation leads to its inhibition of ubiquitination and suppresses apoptosis. NFκB is inhibited and regulated by s-nitrosylation of p50. Nitrosylation activates matrix metalloproteases in pathological processes. The nitrosylated proteins can be identified by biotin switch method, fluorescence method, proteomic method. (121).

3.15 NITRIC OXIDE AND INFLAMMATION:

Under physiological conditions, nitric oxide has anti-inflammatory effect whereas in pathological conditions, it has pro inflammatory effect and induces inflammation. Nitric oxide plays a key role in the pathogenesis of inflammation. Several studies have been carried out to elucidate the role of NO in inflammatory conditions at both acute and chronic state. The pro-inflammatory effect of nitric oxide includes oedema, vasodilation, cytotoxicity and cytokine

mediated process leads to tissue injury (122). It influences many aspects of inflammatory cascade in affected tissues. This can be evaluated by the increased expression of inducible nitric oxide synthase and overproduction of sustained nitric oxide.

The inflammatory conditions in which nitric oxide has an impact includes ischemia reperfusion tissue injury, chronic state of gut inflammatory diseases like ulcerative colitis and crohns, rheumatic diseases, orthopaedic diseases, atherosclerosis and systemic lupus erythematosus, cancer, chronic liver diseases, central nervous system pathologies, stroke, huntington disease, parkinson disease and amylotrophic lateral scelerosis (123).

Nitric oxide mediated inflammatory mechanisms of tissue injury includes activation of NFκB inflammatory pathway (124) (Figure: 3.24), activation of hypoxia inducible factors, regulation of leukocyte recruitment, regulating mitochondrial biogenesis and disturbing T cell activation, cell signaling pathways like cAMP, cGMP, MAPK and JAK/STAT in immune regulation (125).

3.16 NFκB INFLAMMATORY PATHWAY:

Inflammation is a process in which body deals with the injury in reponse to stimuli. The stimuli may be foreign bodies, toxins, microorganisms and chemicals. Inflammation will progress by pro-inflammatory cytokines (TNFα, IL-1, IFNgamma, IL-12, IL-18) and resolved by anti-inflammatory cytokines (IFNα, IL-4, IL-10, IL-13, TGFβ) (56).

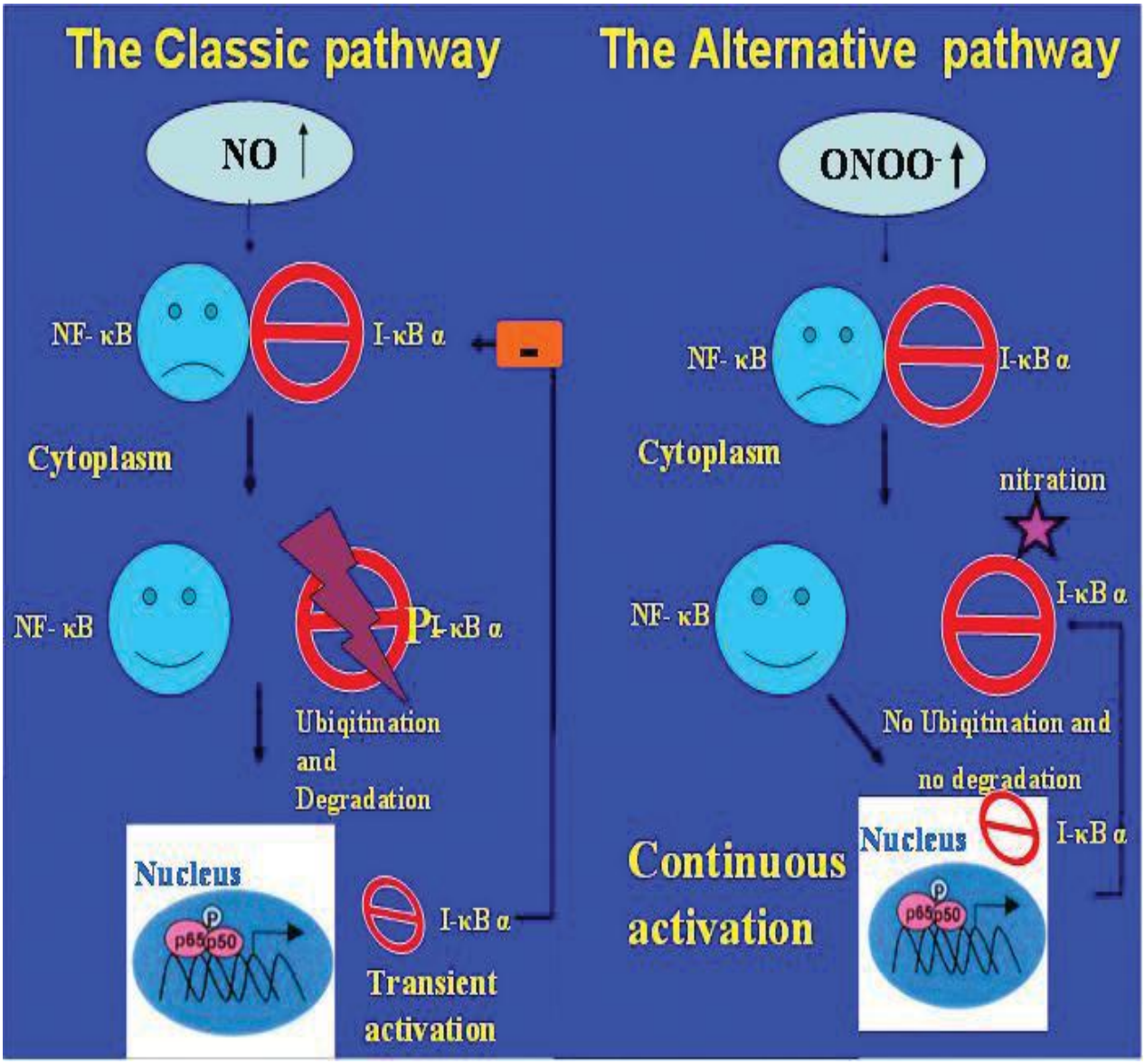


Figure: 3.24 – Classical and alternative pathway of nitrioxide and peroxynitrite mediated NFκB signaling pathway (124)

3.16.1 NF-kappa B:

NFκB has been studied extensively since its first description by (126) for its role in immunity and stress responses. As a major transcription factor and a first responder to harmful cellular stimuli, NF-κB plays a central role in inflammation through its ability to induce transcription of proinflammatory genes (127). NF-κB controls the activity of numerous genes crucial for immunity,cell death, cell survival , angiogenesis , cell proliferation, inflammation, and stress responses, including TNFα, NOS II, COX-2, cPLA2, VCAM 1, ICAM1, cyclinsD1 and E, Bcl2, surviving etc. (128) (129) (Figure: 3.25 and 3.26). Reactive oxygen and nitrogen intermediates, and cytokines such as TNF are thought to be important in activation of the redox-sensitive transcription factor, NF-κB (128) (129) (130) (Figure: 3.27 and 3.28). Studies have shown that tyrosine nitration triggers dissociation of IκB from NuclearFactor-κB (NF-κB), which results in activation of this pathway that plays an important role in cancer and inflammation. Tyr-66 and Tyr-152 of p65 subunit of NFκB have been shown to undergo nitration (111, 131).

NFκB is normally stabilized in the cytosol when bound to its inhibitor protein, IκB to form p50/p65 IκB complex. Activation of the IκB kinase complex by numerous stimuli leads to the phosphorylation of IκB, causing its dissociation from the NF-κB heterodimer and subsequent degradation by the proteasome leaving NFκB available for translocation to the nucleus and activation of target genes (124) (Figure: 3.29). Some target genes for NFκB include pro-inflammatory cytokines such as, cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF-α), iNOS, MMP 9, HO-1, interleukin (IL)-1β and IL-6, which in turn lead to tissue injury and apoptosis. Persistent activation of NF-κB signaling pathways is often associated with chronic

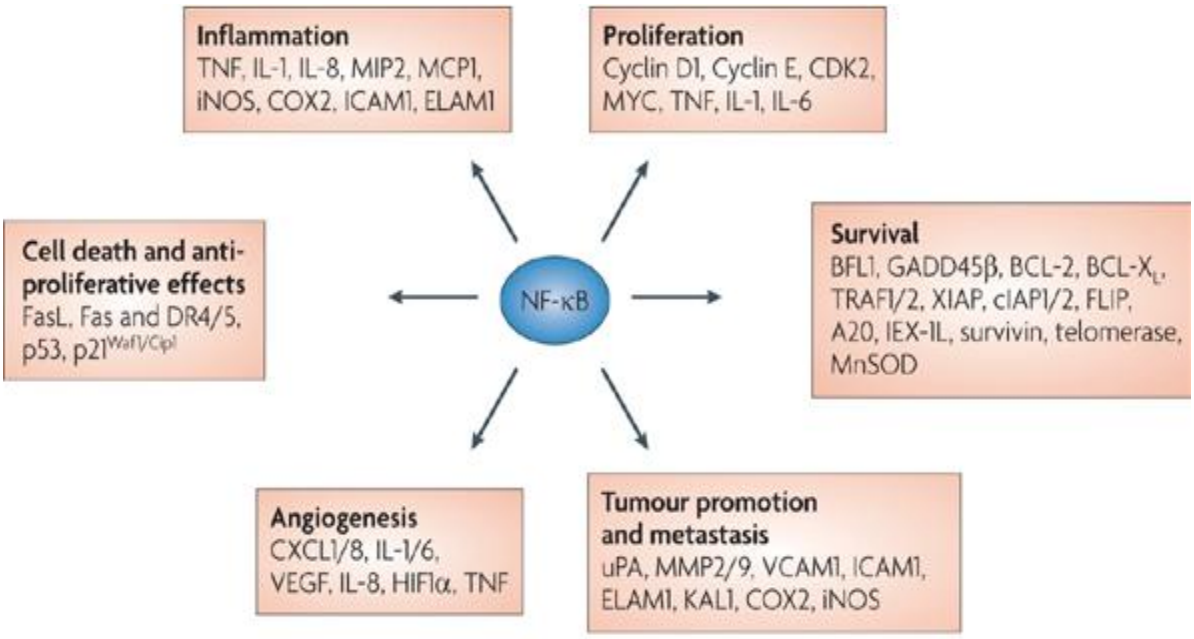


Figure: 3.25 – Target genes of NF-κB (128)

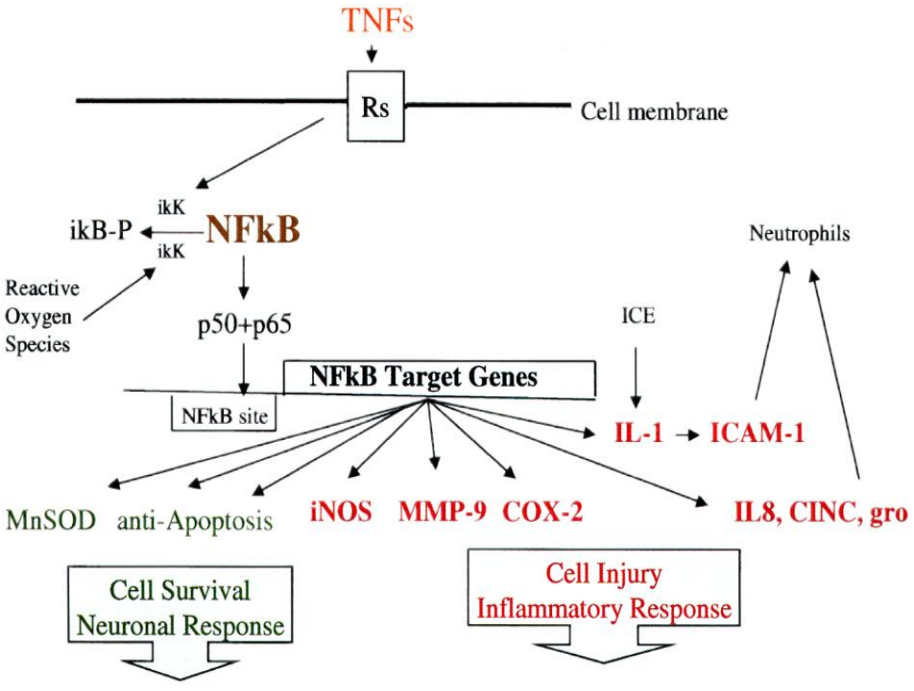


Figure: 3.26 – TNF induced NFκB activation and its downstream proinflammatory target genes (129)

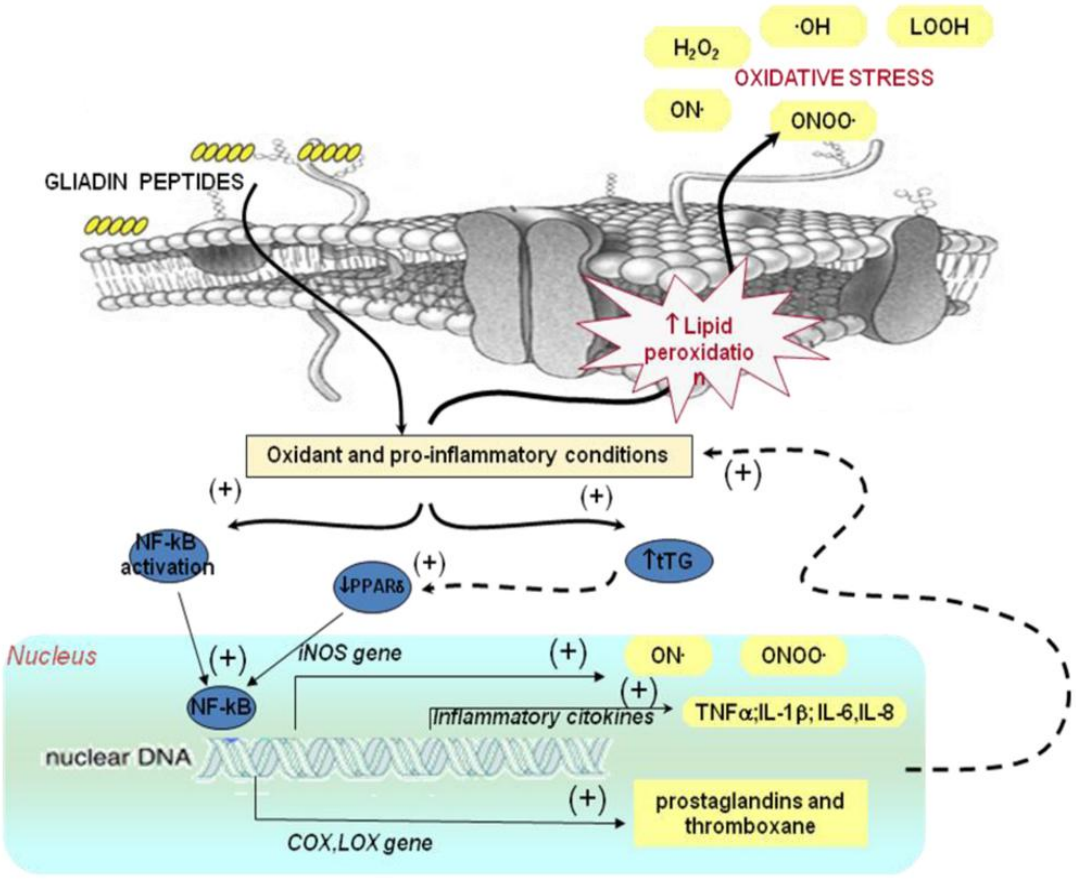


Figure: 3.27 – Regulation of NFκB by ROS and RNS in inflammatory signaling (128)

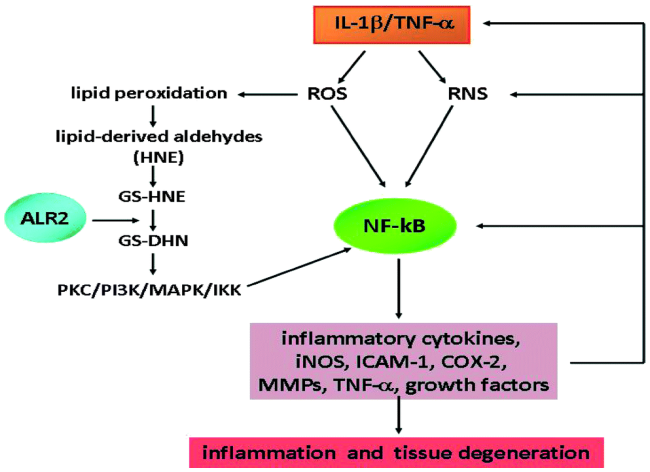


Figure: 3.28 – Crosslink of ROS, RNS and NFκB signaling pathway during inflammation and tissue degeneration (129)

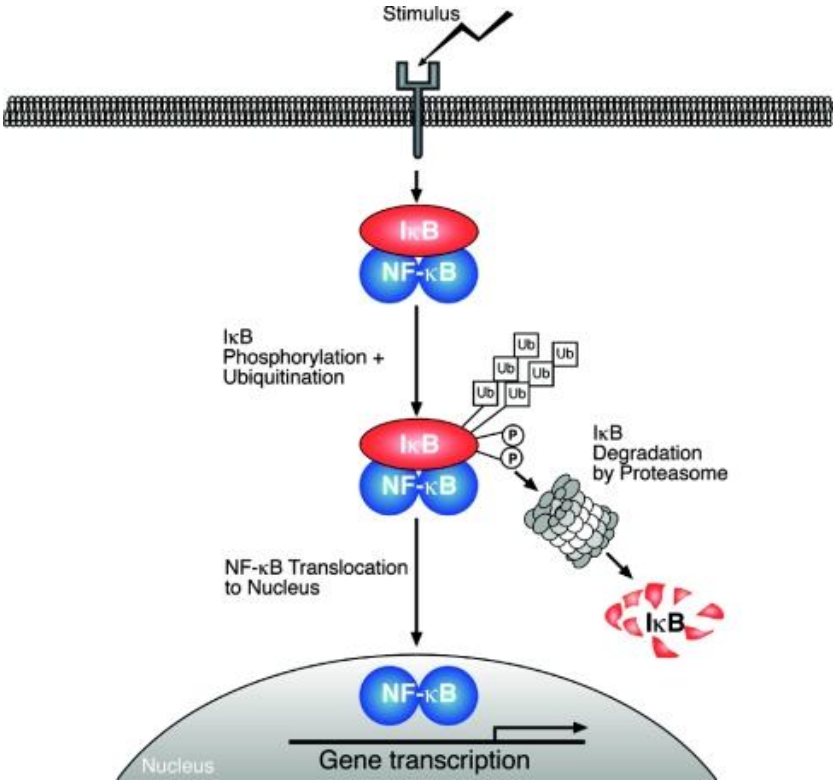


Figure: 3.29 - Activation of NFκB (124)

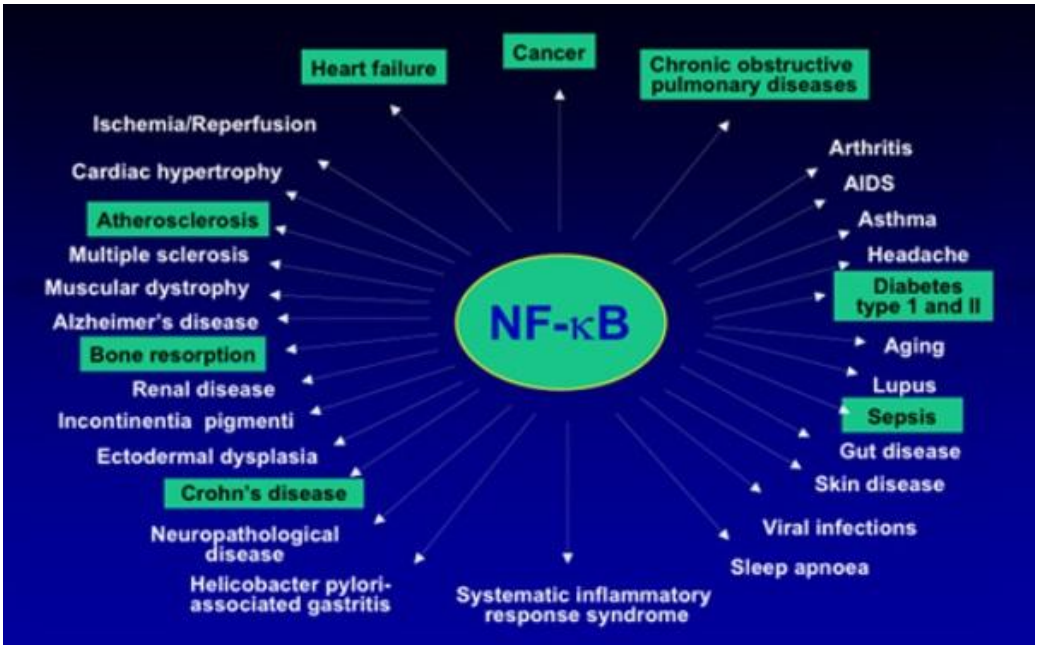


Figure: 3.30 – Role of NFκB in various disease conditions (132)

inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and asthma (180) (132) (Figure: 3.30)

NFκB and small intestines

NF-κB has multiple, often opposing functions in the intestine. NF-κB activity is of particular importance for maintenance of epithelial barriers, but it was also proposed that NF-κB activation in epithelial cells can lead to production of inflammatory chemokines that recruit immune cells to the tissue, thereby initiating an inflammatory amplification cascade (133). Antiapoptotic actions of NF-κB in intestinal epithelial cells dominate tissue responses to many acute inflammatory and injurious challenges, whereas proinflammatory and cell survival functions of NF-κB in macrophages and T cells govern activation of NF-κB in numerous inflammatory processes of the gastrointestinal tract. Induction of this transcription factor has been reported in Helicobacter pylori gastritis, inflammatory bowel disease, and pancreatitis, intestinal ischemia and necrotizing enterocolitis and obstruction and chronic intestinal inflammation (134-135).

3.16.2 TNF alpha:

Tumor necrosis factor alpha (TNF-α) is an adipokine expressed during inflammation. It is an inflammatory cytokine secreted by monocytes, macrophages, neutrophils, adipose tissue, endothelial cells, fibroblasts, neurons, mast cells, T cells and NK cells upon stimulation by lipopolysaccharides and interleukin-1 (136). The biological effect of TNF-α depends on the concentration, cell exposure to pathogens and presence of mediators. It has a role in beneficial processes like immunity, host defense, tissue homeostasis and in pathological conditions occurs due to shock and inflammation (137). TNF-α exerts its effect by binding to its TNF receptor

superfamily as a trimer. The cell membrane receptors for TNF-α are TNFR1 (55kDa) and TNFR2 (75kDa). These receptors have similar structure in extracellular domain where as they differ in intracellular domain. TNF has dual signal transduction function either in cell survival or cell death (138). TNF activates the nuclear transcription factors such as AP-1, IRF-1, IRF-2, and NF-κ B. TNF-α is capable of inducing iNOS expression in various cells. But the direct target of post translational TNF signaling cascade of cellular event identified so far is the activation of NF-κB (139). TNF-α up-regulates the transcription of chemokine genes and hence its own synthesis through the activation of NF-κB. During acute inflammation, TNF-α is responsible for diverse cell signaling events leading to apoptosis or necrosis (140).

TNF and small intestines

Claud, et al., (141) have previously demonstrated that intraperitoneal TNF-α induces intestinal epithelial NF-κB activation. Bertrand *et al*. (142) demonstrated that NSAID induced local production of TNF-α in the small intestine and this event occurred before the elevation of NO production and MPO activity as well as lesion formation

TNF-*α* released early after an inflammatory stimulus, can initiate the infiltration of inflammatory cells into the intestine by activating ICAM-1 and other adhesion molecules (143). TNF is reported to play vital role in various models of GI injury including radiotherapy-induced sub-acute damage (144) and chemotherapy induced mucositis (2). TNF-*α* is a crucial cytokine involved in the pathogenesis of mucositis, which amplifies the NF-*κ*B signal and initiates mitogen activated protein kinase (MAPK) pathway (145).

3.16.3 COX:

Cyclooxygenase (COX) is an enzyme essential for prostaglandins synthesis from arachidonic acid. It was first isolated and purified in 1976. There are two isoforms of COX referred as COX-1 and COX-2. Both the isoforms have similarity in structure and catalytic activity whereas have different biochemical mechanisms, stability, and translational efficiency. COX-1 is constitutively expressed in all cell types where as COX-2 is an inducible isoform expressed during inflammatory conditions. Recently a third isoform found is COX-3 made from COX-1 but retains intron 1 in its mRNA. This COX-3 is not relevant to mice and humans (146). Cyclooxygenase play a major role in renal function (vascular tone and sodium homeostasis), function of nerve, brain, ovarian uterine and in maintaining gastrointestinal integrity. The disease conditions where COX has its adverse effects are local tissue injury, inflammation, pain, fever, alzheimers disease, arthritis, and cancer and the NSAIDs inhibits activity of COX (147). A novel link among $cPLA_2\alpha$, iNOS, and COX-2, which form a multiprotein complex leading to $cPLA_2\alpha$ S-nitrosylation and activation has been demonstrated (148).

The products of the COX pathways and lipoxygenase pathways, and their effects on the tissues are shown in Figure: 3.31 and 3.32 (149). The effect of NO and peroxy nitrite in the activation of COXs is shown in Figure 3.33 (150).

COX and the small intestine

The COX enzymes play significant roles in the gut defense and are key for intestinal epithelium maintenance. COX, the enzyme that catalyzes the first two steps in the biosynthesis of the prostaglandins from arachidonic acid, exists in two isoforms. COX-1 is constitutively expressed throughout the GI tract and, at least in the absence of damage or inflammation, is the major

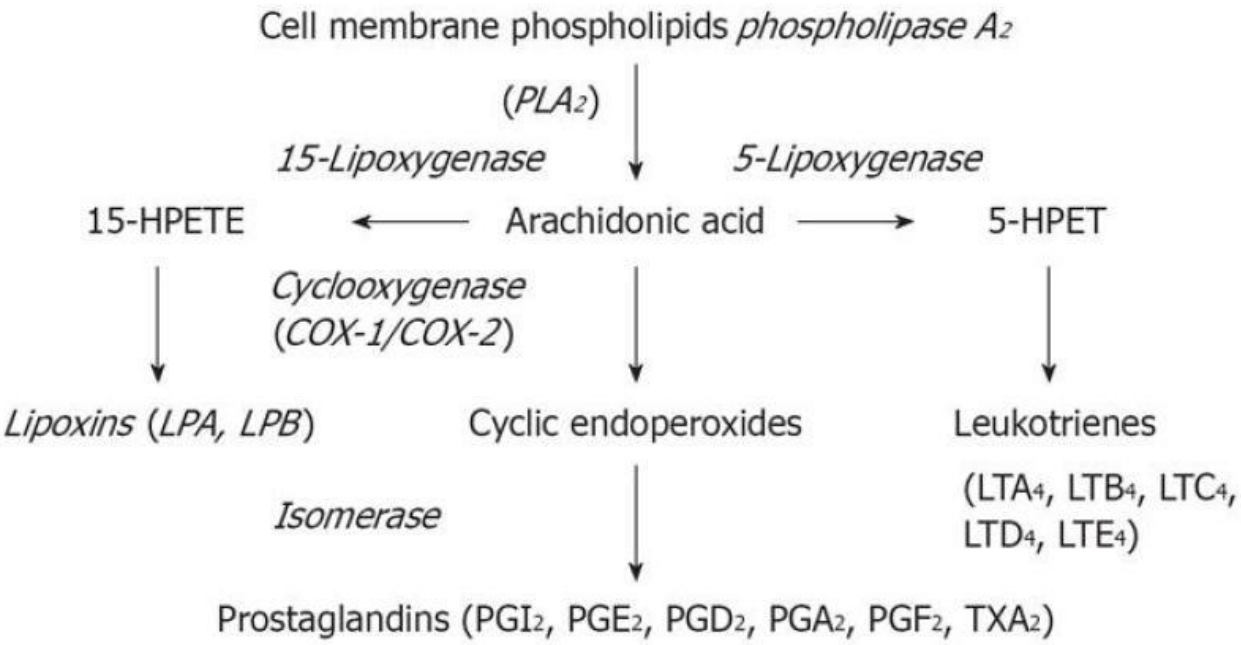


Figure: 3.31 – cyclooxygenase activation pathway (149)

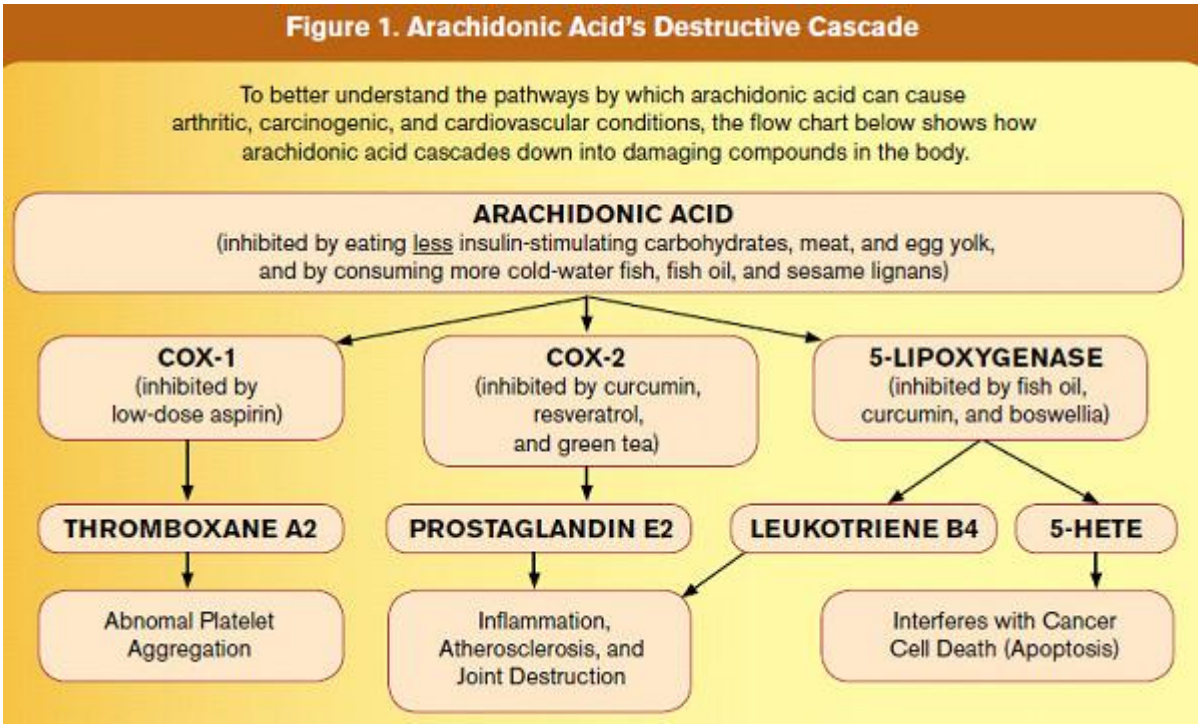


Figure: 3.32 Cascade system of arachidonic acid (149)

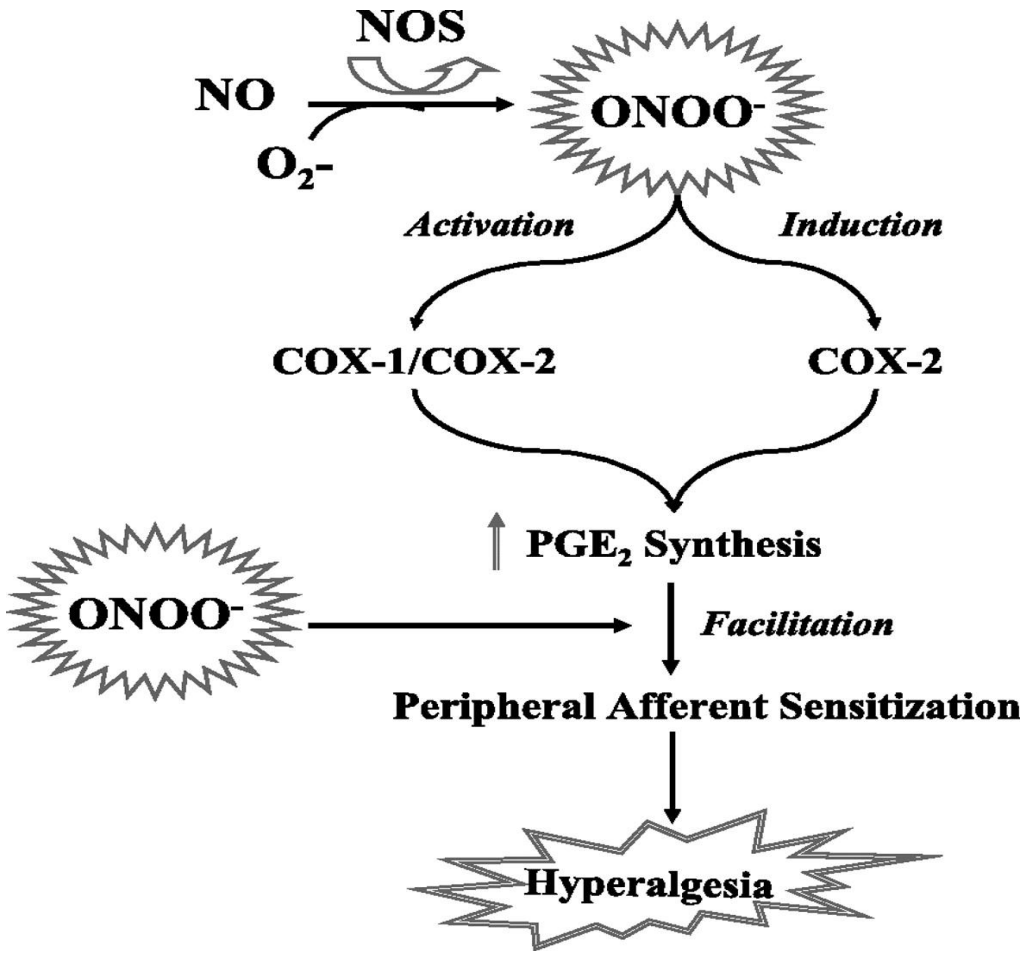


Figure: 3.33 – PON mediated activation of cyclooxygenase (150)

source of prostaglandin synthesis in these tissues (151). The inducible form, COX-2, is either undetectable or expressed at extremely low levels in the healthy GI tract of humans and various animals. However, in response to various proinflammatory stimuli, COX-2 is rapidly induced (152) leading to increased prostanoid production. Prostanoids play an important role in intestinal epithelial homeostasis and inflammation and are known to protect intestinal cells from apoptosis (153-154).

Overactivation of COX-2 and production of PGs have been reported in various conditions of intestinal inflammation such LPS induced GI injury (152), NASID induced enteropathy (155), animal models of necrotizing enterocolitis NEC (156) and inhibition of COX-2 by different agents reduce PG production and intestinal inflammation. In both humans and animal models, the role of COX-2 in GI intestinal mucositis is supported by the observations that the administration of COX-2 specific inhibitors prevents GI injury and inflammation. Patients taking a supra therapeutic dose of rofecoxib or celecoxib had significantly lower rates of GI-related adverse events than those taking a nonselective NSAID (146).

3.16.4 HSP70:

HSP70 proteins are the molecular chaperones which assist protein folding processes. Expression of heat shock protein (HSP) is an endogenous mechanism by which living cells adapt to stress. Induction of HSPs protects cells not only from damage due to heat but also from damage due to oxidative injury and cytokine-mediated cytotoxicity (157). Interestingly, the same oxygen-free radicals, such as superoxide, also induce the expression of various HSPs which take part in the defense against oxidative stress. HSPs are expressed in many tissues, including heart, brain,

muscle, lung, kidney, liver and intestinal and colonic epithelium (157). These highly conserved molecules are responsible for maintaining adequate protein folding and influencing the degradation of proteins and cell repair processes after damage (158). Furthermore, HSPs are involved in the modulation of immune responses, autoimmunity, cell signalling, cell proliferation, apoptosis, and tumour cell differentiation and invasion (157).

Based on their molecular weight they can be classified into six major families: small HSPs (molecular weight <30 kDa), HSP60s, HSP70s, HSP90s, HSP100s, and other non-ubiquitous HSPs. HSPs can act as "danger signals" for the immune system at the sites of tissue injury (159). HSP70 can also promote cell survival by inhibiting the mitochondrial translocation of Bax and subsequent release of cytochrome c and activation of caspase-9 and -3, an intrinsic apoptotic pathway that is initiated by intracellular stress signals (160). Induction of Hsp70 protects cells not only from damage due to apoptosis induction but also from damage due to oxidative injury. Furthermore, only cells overexpressing Hsp70 were found to be protected from both ROI- and TNF induced cytotoxicity (160).

High levels of NO exposure induce protective stress responses, stimulating Hsp70 expression. NO donor-induced Hsp70 expression inhibits both ROI production and lipid peroxidation. Accumulated data suggest that low levels of nitric oxide may contribute to apoptotic pathway suppression by the upregulation of Hsp70 and that interaction is an early line of defense for protecting cells from death (161).

HSP70 and small intestine

Heat shock proteins (HSPs) play an important role in mucosal defense of gastrointestinal tract. Studies have shown that the expression of HSPs in the small intestine is normally negligible (162), but the expression of HSP25 and HSP70 is markedly increased in the intestinal epithelial cells under stress (163). The predominant localization of HSPs in intestinal epithelial cells suggests their primary role is in maintaining the integrity of the enterocyte layer, as demonstrated by Kojima *et al* (164).

3.16.5 HO-1:

Heme Oxygenase (HO)-1, known as HSP 32, is ubiquitously distributed in mammalian cells and tissues and is potently induced by various stimuli, such as oxidative stress and pathological conditions. Heme oxygenase-1 (HO-1) is the inducible of three isoforms of HO, the enzyme that catalyzes heme breakdown, generating equimolar amounts of bilirubin, free iron, and carbon monoxide (CO) (165). Many studies have shown that HO-1 is involved in a variety of regulatory and protective cellular mechanisms as a stress-responsive protein (165). Heme oxygenase -1 is inducible by a variety of oxidative stress and is thought to play an important role in the protection of tissues from oxidative injuries. In addition, HO-1 is also reported to be one of the target of NFκb which is plays a pivotal role in inflammation. It is important to mention HO-1 gene expression is not only regulated via NF-κB but that HO-1 can modulate the activity of NF-κB in various cell types (166).

HO-1 and small intestines

HO-1 is expressed constitutively in normal gastric, intestinal, and colonic mucosa (167), and is up-regulated in their inflamed tissue (168). HO-1 induction plays a fundamental role in

protecting mucosal cells of the intestine from oxidative damages (169). Chung and coworkers (170) demonstrated that there is an exaggerated lethality in HO-1 homozygous knockout mice (KO, *Hmox*1$^{-/-}$) following sepsis due to gross tissue destruction and loss of bowel integrity in the ileum and colon. Overexpression of HO-1 in smooth muscle cells and myofibroblasts of blood vessels was shown to increase bacterial clearance by enhancing phagocytosis and the endogenous antimicrobial response, thus improving survival.

Interestingly, expression of HO-1 is usually increased in gastrointestinal inflammation and injury. This was shown in radiation enteritis (171), inflammatory bowel disease (IBD) (172) of animal models or patients suggesting that the activation of HO-1 may act as an endogenous defensive mechanism to reduce inflammation and tissue injury in the intestinal tract.

3.16.6 MMP:

Matrix metalloproteinases (MMP) are structurally related proteins responsible for the metabolism of extracellular matrix. There are more than 24 human MMPs identified and they are collagenases (MMP-1,-8,-13,-18), gelatinases (MMP-2,-9) stromelysins (MMP-3,-7,-10,-11), elastase (MMP-12) and others (MMP-19,-20,-23,-26,-27,-28). These are secretory enzymes found in macrophages, monocytes, neutrophils and cancer cells (173). A subset of MMPs known as membrane type MMPs (MMP-14,-15,-16,-17,-24,-25) are not secreted but remain on cell surfaces (173). MMPs are involved in the tissue remodeling of cell membrane during growth and clearance during cell death. They are also involved in ovulation, embroyonic growth and differentiation, parturition, turnover of gastric extracellular matrix. The MMPs particularly collagenases, MMP-2 and MMP-9 participate in the degradation of extracellular components and

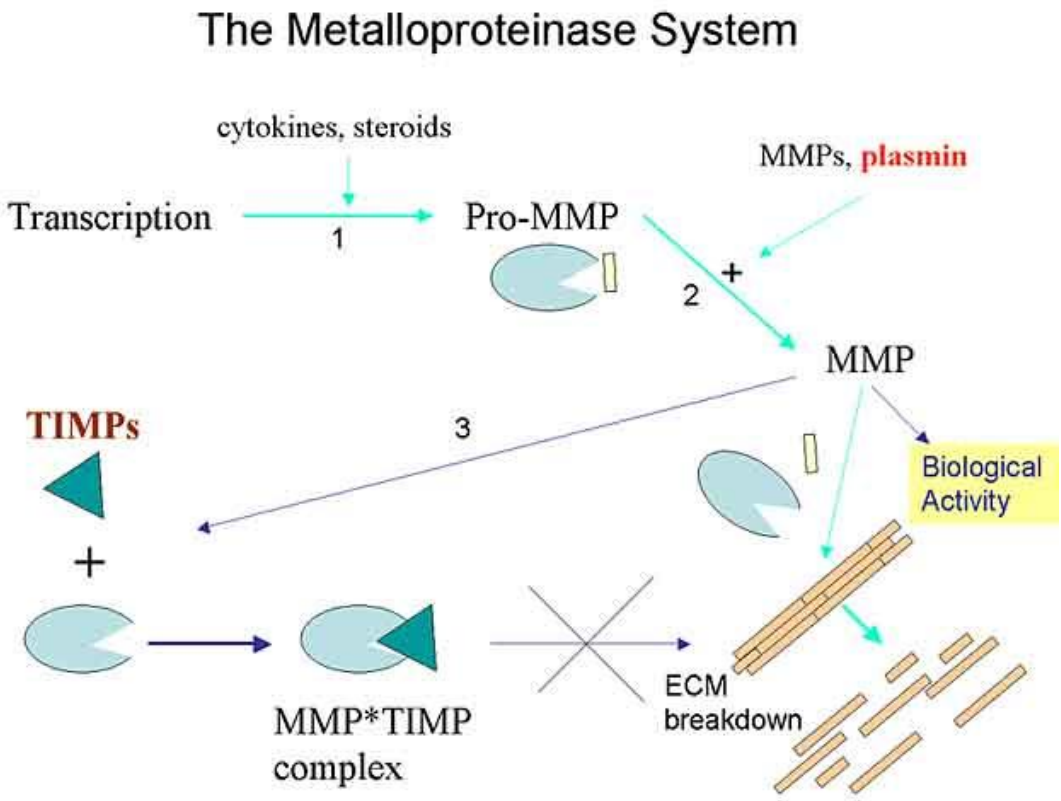


Figure: 3.34 – Biological function of MMPs (175)

highly expressed in inflammatory bowel diseases (174). MMP genes are transcriptionally responsive to the cytokines such as TNF-α, IL-1, mitogens and growth factors. The enzyme activity of MMPs are regulated by its inhibitors TIMP (TIMP-1,-2,-3,-4)(175). (Figure: 3.34)

MMPs and small intestines

In recent years matrix metalloproteinases (MMPs) have been implicated as being key mediators in the development of mucositis possibly through inflammatory pathways, alterations in extracellular matrix composition, adhesion molecules and tight junctions (176-177). MMPs can be induced by the activity of pro-inflammatory cytokines such tumor necrosis factor-α, interleukin (IL)-1, IL-6 and IL-8 and NFκB that bind to specific elements on MMP gene promoters (178).

MMP2 and 9 appear to have apposing effects on the intestines, while MMP 2 has anti-inflammatory effect and MMP 9 has proinflammatory effect (179). It has been hypothesized that MMP-9 plays an important role in both initiation and termination of the inflammatory response. It is suggested that MMP-2 expression during colitis served to protect from the development of inflammatory response likely through its effect on the epithelial barrier. On the other hand, overexpression of MMP-9 impaired wound healing in cultured intestinal epithelial cells in vitro and modulated colonic epithelial differentiation, suggesting that MMP-9 mediates inflammatory response and/or tissue damage (179-180).

3.17 APOPTOTIC PATHWAY:

Apoptosis is a programmed cell death essential in normal tissue homeostasis especially skin, immune system and gastrointestinal tract. It is characterized by distinctive morphological and biochemical changes like cellular shrinkage, chromatin condensation, nuclear component fragmentation, endonuclease activation and alterations in cytoplasmic enzymes (181). Apoptotic cell death is characterized by the ATP-dependent activation of cysteine proteases (caspases) (182). By contrast, cell death primarily through inhibition of mitochondrial respiration and loss of ATP production is normally classified as necrotic (183). Recently, a large number of studies have associated mitochondrial dysfunction caused by ROS/RNS lead to both accidental cell death (necrosis) and programmed cell death (apoptosis) (184-186).

Peroxynitrite can trigger the release of mitochondrial pro-apoptotic factors and trigger cytochrome-c-dependent apoptosis in the cytosol through the peroxynitrite dependent oxidation of permeability transition pore components (108). Mitochondrial outer membrane permeabilization (MOMP) leads to the release of cytochrome c (cyt c) into the cytosol. Cyt c is a key activator of caspases. Although peroxynitrite may cause necrosis by de-energizing the mitochondria, it can also lead to apoptotic cell death via activation of the caspase machinery. MOMP and the resulting efflux of proapoptotic signaling molecules occur universally following peroxynitrite exposure. Cytosolic cyt c acts in concert with apoptosis activating factor-1 (Apaf-1) to activate procaspase 9. Active caspase 9 is an initiator caspase, which in turn activates effector caspases, such as caspases 3 and 7. Active effector caspases execute apoptosis by dismantling key proteins and cellular structures. Caspase-3, one of the key executioners of apoptosis, is essential for DNA fragmentation and the morphological changes associated with

apoptosis. Caspase mediated apoptotic cell death is accomplished through the cleavage of several key proteins required for cellular functioning and survival. PARP-1 is one of several known cellular substrates of caspases. Cleavage of PARP-1 by caspases is considered to be a hallmark of apoptosis (187).

3.17.1 CYTOCHROME-C:

Cytochrome c is a specific electron transfer mediator in mitochondrial respiration process. It is present loosely/tightly bound to inner membrane of mitochondria by association with cardiolipin. Disruption of cytochrome c from cardiolipin and permeabilization of outer membrane by bax is responsible for the release of the protein (188). Cytochrome C oxidase, a terminal respiratory chain enzyme found in mitochondria and catalyze transfer of electron from cytochrome c to oxygen molecule and later reduced to water (189). This cytochrome c has a role in cell death function beyond respiration process as described above. The release of cytochrome c is also regulated by Bcl-2 (190). The mechanism of release of cytochrome c and its role in apoptosis is shonw in (Figure: 3.35) (191).

3.17.2 CASPASES:

Caspases are endoproteases involved in cell regulatory pathways that control inflammation and apoptosis. These hydrolyze peptide bonds depending on the cysteine residues in active caspase site and aspartic acid in substrate. Based on their role they are classified into two groups. Caspases involved in apoptosis: initiator caspases (caspase-2, -8, -9, -10) and executioner caspases (caspase-3, -6, -7). Caspases will be in inactive form and it requires dimerization or cleavage for activation. The initiator caspases needs dimerization and executioner caspases require cleavage for the activation of apoptotic signaling pathway. Caspase-9 is an essential

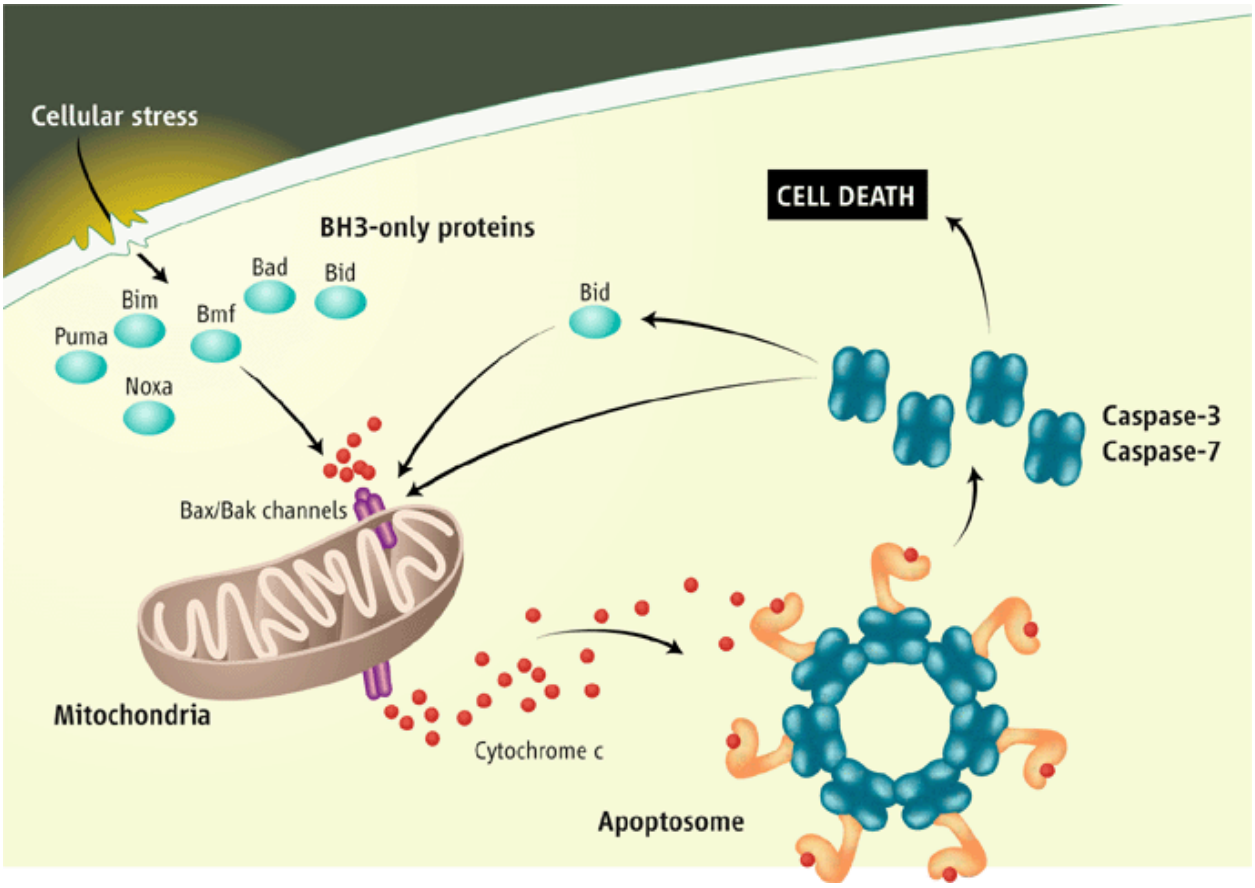


Figure: 3.35 - Mechanism of cytochrome c release and its role in apoptosis (191)

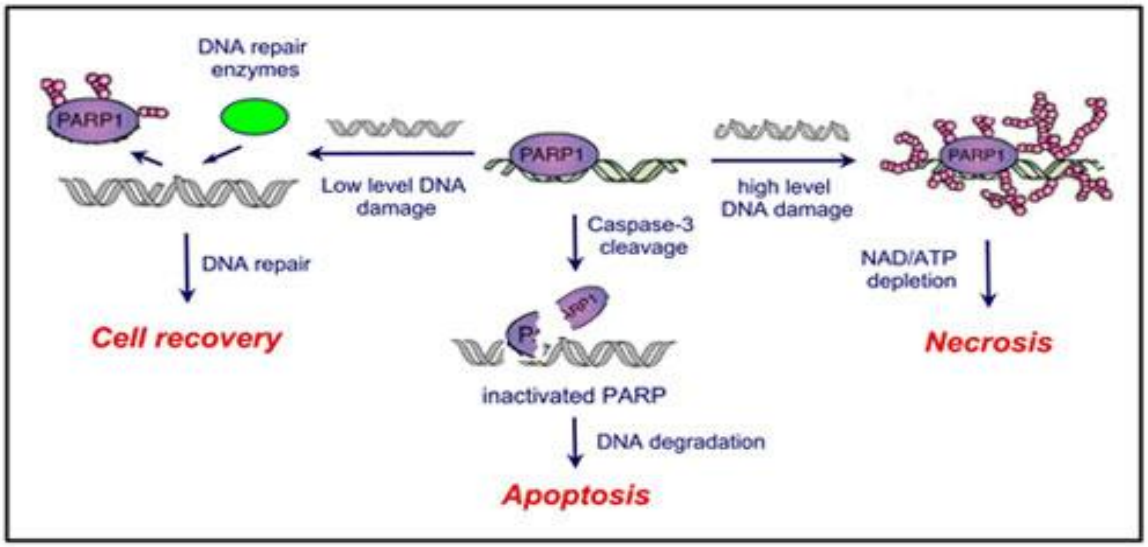


Figure: 3.36 - Role of PARP in cell recovery, necrosis and apoptosis (196)

initiator caspase involved in the mitochondrial mediated apoptotic pathway and activated by apoptosome complex (192). Further the downstream signaling started by activating caspase 3 and inducing cell death mechanism. The caspases processing and activity was regulated at different levels like transcription status (IRF-1, STAT-1), death receptors (FLIP), cytochrome c pathway (apaf-1, Bcl2, Bcl-xl), apoptotic inhibitor proteins (XIAP, cIAP-1, cIAP-2)(193).

3.17.3 BCL2:

BCL-2 family protein regulates apoptosis as both inducer and inhibitor of cell death. It particularly regulates mitochondria mediated intrinsic signaling pathway. BCL-2 has a role in normal physiology of cell related to mitochondrial dynamics and energetics, autophagy, calcium homeostasis, and others. These BCL2 family proteins govern MOMP and have role in apoptosis as either pro-apoptotic (BAD, Bax, Bak, Bok) or anti-apoptotic (Bcl-2, Bcl-XL, Bcl-w, MCL-1). The cellular stress causes changes at transcriptional level and dictates mitochondrial outer membrane to induce BH2 and effector protein interaction and allow release of soluble protein cytochrome c from mitochondrial intermembrane space to cytosol, recruitment of APAF-1 and activation of caspases causing cell death (194).

3.17.4 PARP:

Poly ADP ribose polymerases (PARPs) are the enzymes involved in the process of poly ADP ribosylation by transferring ADP ribose to proteins. There are around 18 members in PARP family which have role in cell proliferation and cell death pathways. The cellular substrates are mostly nuclear proteins involved in the metabolism of nucleic acid, modulation of chromatin, DNA synthesis and repair mechanisms. Damage in the DNA cause increase in PARP-1 levels

and activity as it is involved in the repair mechanism (base excision repair) during single strand breaks in DNA molecule. Other mechanism in which PARP-1 has its effect are mitochondrial dysfunction, NAD+ depletion and AIF translocation from mitochondria to nucleus (195). PARP-1 has two modes in cell death: apoptosis and necrosis. Overactivation of PARP-1 leads to depletion in energy and necrosis. Caspase 3 cleaves full length parp-1 (116 kda) to 2 apoptotic fragments; C terminal catalytic fragment (87 kda) and N-terminal fragment (25 kda) l. The DNA dependent catalytic activity of C terminal fragment of parp-1) is lost due to the cleavage where as N-terminal fragment (25 kda) binds to DNA and completely inhibits parp-1 activity. Thus cleavage of parp-1 prevents DNA repair survival mechanism, blocks necrosis by energy depletion and promotes apoptosis (196). (Figure: 3.36)

3.18 NITRIC OXIDE AND APOPTOSIS:

Nitric oxide has been reported in previous research work as both pro-apoptotic and anti-apoptotic molecule. Under physiological conditions nitric oxide has anti-apoptotic effect where as the pathophysiological role depends on its concentration and coexistence of noxious agents. During pathogenic condition nitric oxide induces cytotoxicity and acts as an effector of apoptosis by activation of intrinsic apoptotic pathway leading to DNA fragmentation (197).

Nitric oxide induces apoptosis by binding to cytochrome c in mitochondria leading to superoxide formation and peroxynitrite generation in mitochondria. Nitric oxide and peroxynitrite damage or inhibit the mitochondrial respiratory complexes, aconitase, creatine kinase, MnSOD, mitochondrial membrane transient permeability, increase mitochondrial swelling and cause release of cytochrome c from mitochondria (198). Nitric oxide increases the bax/bcl2 rate,

inhibits bcl2 expression, triggers caspase activation and cause apoptosis. The nitric oxide also have protective effect on apoptosis by stimulation of protein kinase, modulation of bax/bcl2 family and induction of hsp70 (199).

The MnSOD protein nitration and nitrosylation modification also triggers apoptosis by inducing the membrane permeability of mitochondria (108). Peroxynitrite causes inhibition in mitochondrial electron transport. Thus mitochondria act as intracellular loci for peroxynitrite toxic effects under pathological conditions (200). Denitrosylation of caspase 3 induce the activation of caspase cascade mediated apoptosis where as s-nitrosylation of cystein residue of caspase 3 cause anti-apoptotic effect (201).

At physiological concentration the anti-apoptotic effect of nitric oxide also includes inhibition of mitochondrial membrane trasition pore and release of cytochrome c, activation on HSP70, effect of TNF alpha and inhibiting caspase activation, upregulation of cyclooxygenase 2 or hemeoxygenase (202). Thus, nitric oxide act as modulator of both proapoptotic factors like cytochrome c release, p53 upregulation, alteration in Bcl2 family as well as anti-apoptotic factors like hsp70, HO or COX-2 and inhibition of caspases (203) (204) (Figure: 3.37)

Apoptosis and small intestines

Apoptosis is an inherent protective mechanism used by cells to regulate proliferation and occurs at a low level in the healthy, normal small intestine. Thus, apoptosis is an important factor in gastrointestinal physiological cell renewal, which can be triggered by noxious stimuli such as trauma and ischemia (205-206). Previous studies have demonstrated that apoptosis of the

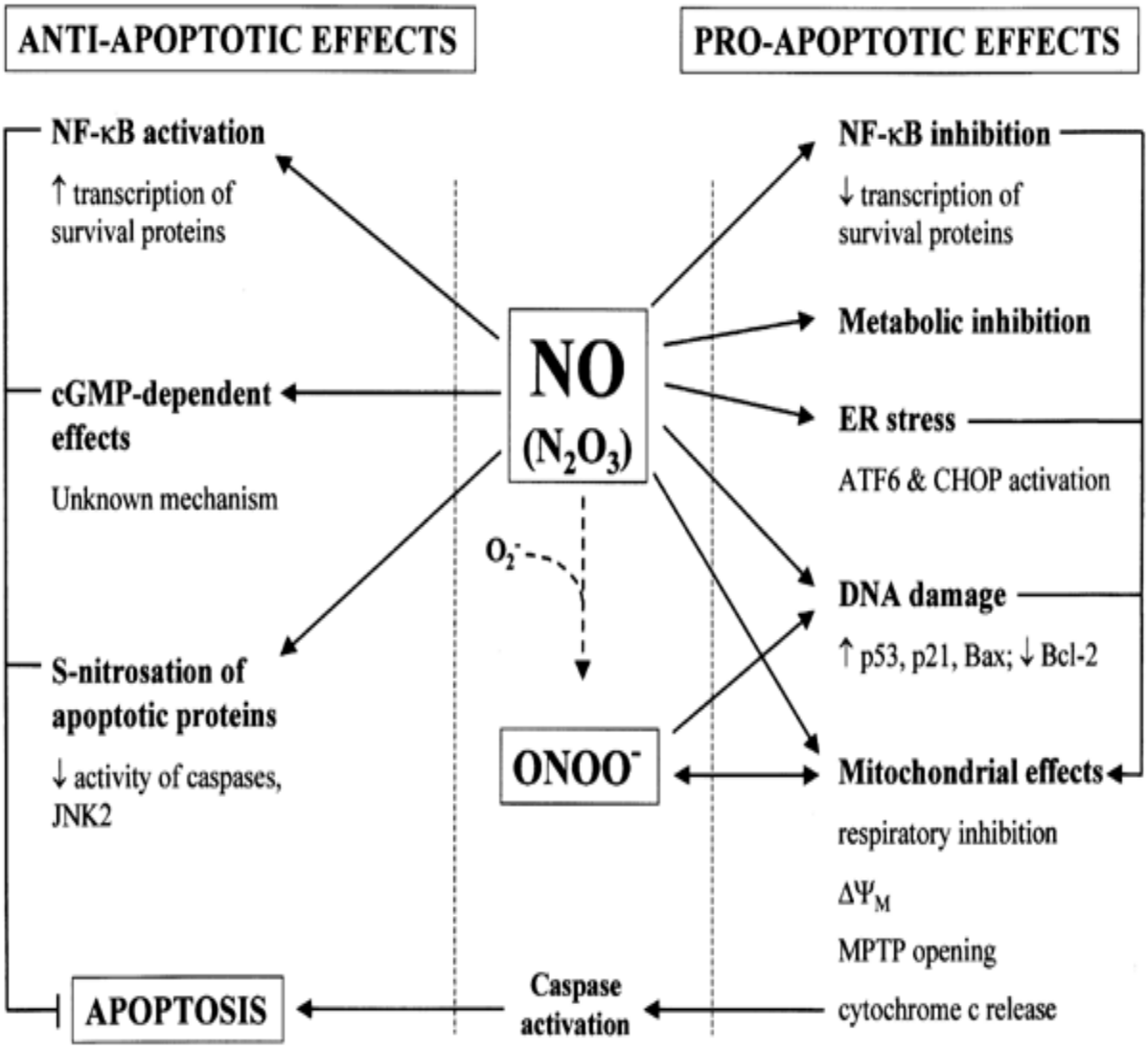


Figure: 3.37 – Role of nitric oxide in apoptosis (204)

intestinal epithelial cells plays an important role in the gut barrier damage and increases permeability of the intestinal epithelium, leading to possible translocation of intraluminal microbes and bacterial toxins (143, 207).

3.19 NOS INHIBITORS:

Selective inhibition of iNOS has a beneficial role against inflammation. The strategies to achieve NOS inhibition includes targeting calmodulin or tetrahydrobiopterin cofactor requirement, targeting L-arginine substrate requirement and by use of pharmacological agents developed specifically against isoforms of NOS (208). iNOS inhibitors such as N-nitro-L-arginine, L-Nitro arginine methyl ester, 7-nitroindazole, 1-2-trifluoromethylphenyl imidazole, S-ethylisothiourea, S-methylisothiourea and aminoguanidine were selectively inhibits iNOS at their particular concentrations (209).

3.19.1 AMINOGUANIDINE:

3.19.1.1 Introduction:

Aminoguanidine (AG) was prepared more than 100 years ago by the reduction of nitroguanidine in 1892. It can also be prepared by the hydration or hydrolysis of guanidine related compounds and so have some common effects of hydrazines also. It has a hydrazine group and a guanidino moiety in its structure (210). (Figure: 3.38)

3.19.1.2 Biological effects of aminoguanidine:

AG structurally similar to L-arginine and thus competitively inhibits nitric oxide synthase. Aminoguanidine inhibits selectively the cytokine inducible nitric oxide synthase isoform which

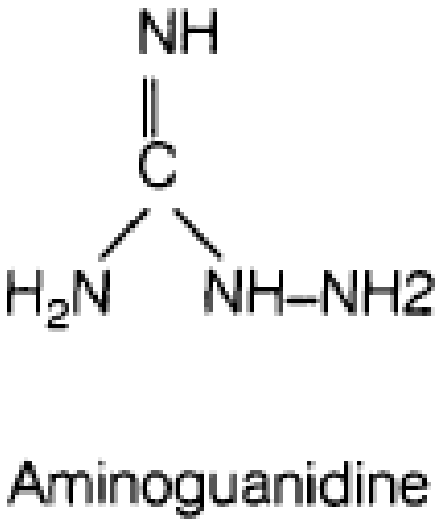


Figure: 3.38 – Structure of aminoguanidine (210)

is responsible for the production of excess nitric oxide in disease states. This iNOS inhibition leads to decreased generation of nitric oxide in tissues (211). Aminoguanidine was first identified as selective iNOS inhibitor and it has fifty fold more effect inhibition on iNOS enzyme activity than endothelial and neuronal NOS (212). Thus, AG markedly potentiate anti-inflammatory effect in a nitric oxide dependent manner (213).

Two other biological effects of AG have been identified. AG inhibits the formation of advanced glycosylation end products which are highly reactive in pathogenesis of disease states (210). AG has been reported to possess strong antioxidant property (214). It is an effective hydroxyl radical scavenger. AG exhibits a significant dose-dependent effect against free radical damage. Aminoguanidine has been shown to exhibit trapping activity toward lipid-derived aldehydes such as MDA and 4-hydroxynonenal (215). Several studies have shown that AG acts as an antioxidant and can restore the antioxidants in the tissues as well as prevent cellular damage (214). These effects made the molecule as an attractive pharmacological tool.

3.19.1.3 Uses of aminoguanidine:

Aminoguanidine inhibits advanced glycation in chronic diabetic complications (216), inhibits disease as dose dependent manner in autoimmune encephalomyelitis and reduces pathology in spinal cord inflammation, demyelination and necrosis of axon in rats, suppress rapid sleep wave and delay slow wave sleep and improve survival rate and behavioral symptoms in rats, attenuate nitric oxide induced apoptosis and induce proliferation of cells (217), ameliorate endotoxin induced mucosal permeability and prevents dysfunction of gut mucosal barrier in rats (218)

3.20 MTX AND GASTROINTESTINAL INFLAMMATION:

MTX has severe morphological changes in gastrointestinal system. The ultrastructural damage of MTX treated small intestine tissues revealed villus atrophy, crypt cell abscess, shortening of villus, epithelial desquamation, globlet cell depletion, microvillar damage, crypt loss, fused and blunted villi, loss of glandular structure, atrophic intestinal wall (219). The exact mechanism for such adverse effects of MTX on the small intestines is unclear.

3.20.1 Mechanisms proposed for MTX induced small intestinal injury

1. MTX treatment causes alterations in small intestinal epithelium response (21), increased intestinal permeability, mucosal protein, DNA and RNA content (220)
2. Oxidative stress contributes to intestinal toxicity as shown by increased MDA levels (221)
3. High dose of MTX induces ROS generation, loss in mitochondrial membrane potential and activation of caspase 3 in IEC-6 cells causing decreased cell viability and apoptosis (222).
4. Enterocyte apoptosis is caused by increased bax and decreased bcl2 (223),
5. Increased neutrophil infiltration and MPO activity contribute to MTX enteritis (224)
6. MTX induced small intestinal injury is associated with increased nitrite levels and iNOS expression (225)

Recent findings from the work carried out in our laboratory on MTX induced small intestinal injury in rats

1. MTX administration results in increased oxidative stress and depletion of antioxidant enzymes (224),
2. MTX induces nitrosative stress in the small intestines of rats as evidenced by elevated tissue nitrate and nitrotyrosine expression and increase in iNOS expression (25).
3. Neutrophil infiltration and increased MPO activity in MTX treated rat intestines (24)
4. Mitochondrial dysfunction and changes in mitochondrial structure contribute to MTX induced enteritis (23)

Based on this background information, the present study was designed to elucidate in detail the role of iNOS and peroxynitrite in MTX induced small intestinal injury using rat model. In addition we also investgated whether pretreatment with aminoguanidine, a selective iNOS inhibitor ameliorates MTX induced small intestinal damage

CHAPTER – 4

Plan of work

The rat is a suitable model for the study of pathogenesis of gastrointestinal toxicity of chemotherapeutic agents. Adult male Wistar rats with 200 – 250 gms body weight were used for the studies. Gastrointestinal injury was induced in the rats by the administration of 7mg/kg body weight MTX intraperitoneally for three consecutive days. The rats were sacrificed 24hours after of final dose of MTX. Control rats were treated with vehicle alone for 3 consecutive days.

For the intervention studies, aminoguanidine 30mg or 50mg per kg body weight was administered intraperitoneally one hour before the administration of methotrexate for three consecutive days and the rats were sacrificed 24hours after the final dose. The entire length of small intestine tissue was removed from each rat, washed with saline and a piece of duodenum, jejunum and ileum sections were used for histology and histochemistry studies. Mucosa was collected by scraping from the remaining tissue and utilized for biochemical assays, western blot and polymerase chain reaction studies.

BRIEF DESCRIPTION OF EACH STUDY PROTOCOL:

STUDY - I was done to ascertain the role of nitrosative stress in MTX induced enteritis in a rat model. Studies were carried out by the intraperitoneal injection of 7mg/ kg /day of MTX and equal volume of NaCl-NaOH as vehicle for 3 consecutive days. Twenty four hours after the final dose of MTX, rats were sacrificed and the entire length of the small intestine tissue was removed, washed with saline and a piece of tissue segments (duodenum, jejunum, ileum) was stored in neutral formalin for histochemistry studies. The mucosal scrapings of the remaining tissue was collected and used to measure the nitric oxide levels (nitrate and nitrite) by spectrophotometric method. The iNOS and nitrotyrosine protein expression was done by

immunohistochemistry methodand westetrn blot. The mucosa scrapings was collected, stored and used for measuring nitrate, nitrite biochemical assay, iNOS gene expression by RT-PCR, nitration of proteins by immunoprecipitation and western blots.

We also carried out experiments to identify the proteins that may be tyrosine nitrated. For this, the mucosa of control and MTX treated rat intestines were homogenized and the cellular fractions like nucleus, mitochondria, microsomes and cytosol were isolated from the homogenate by ultracentrifugation. SDS-PAGE technique followed by western blot of nitrotyrosine was done using monoclonal anti-nitrotyrosine antibody in all the cell fractions.Coomassie brilliant blue staining was done in each sample fractions to confirm equal loading and non-degradation of samples. Molecular weight of each band was approximately determined using molecular weight marker and the band intensity was quantified using alpha innotech fluorchem SP software. Representative graphs were plotted with the obtained band intensity to identify increase or decrease in the tyrosine nitration of identified proteins.

STUDY - II was done to determine the effect of MTX on the activities of enzymes that have been identified to undergo nitration. Mucosa of control and MTX treated rat intestines were homogenized and the 10% homogenate was centrifuged at low speed to remove the cell debris. The clear homogenate sample was used for the analysis of enzyme activity. The enzymes analyzed were aconitase, creatine kinase, glutamate dehydrogenase, superoxide dismutase, catalase, glutathione-s-transferase, carbonic anhydrase, succinate dehydrogenase, cytochrome-c oxidase, F1-ATPase.

STUDY - III was designed to assess the role of NFκB-iNOS-COX-2-TNF inflammatory signaling pathway in methotrexate induced enteritis. A piece of small intestine segments was fixed in 10% formalin and used for the immunolocalisation of proteins using HRP as a secondary marker, DAB as a chromogen and immunofluorescense technique. Mucosal scrapings was homogenized and used for protein expression by immunoblots and gene expression by RT-PCR technique.

- RNA was isolated from the snap frozen samples and the integrity was assessed by agarose gel electrophoresis, cDNA was constructed and the mRNA gene expression of TNF-α, NFκB, iNOS, COX2 was done by RT-PCR method.
- Immunoblots of whole cell lysates, nucleus and cytosol fractions was used for protein expressions of TNF-α, NFκB, COX2.
- Immunolocalisation of proteins was done in small intestine segments (duodenum, jejunum, ileum) for NFκB, COX2, PLA2 by immunohistochemistry method. NFκB in nucleus expressions was assessed by immunofluorescence method.
- Gene expression of HO-1, HSP70, MMP2, MMP9 was done by RT-PCR.
- Protein expression of MMP-2 and MMP-9 was done by western blot.

STUDY - IV was done to investigate the role of intrinsic apoptotic pathway in methotrexate induced enteritis. Small intestine segments were fixed in formalin and used for immunohistochemistry and immunofluorescence methods for protein expression on tissue sections. Mucosal scrapings was homogenized and utilized for the detection of protein expression by immunoblot and mRNA gene expression by RT-PCR.

- mRNA gene expression of caspase 3 was measured by RT-PCR analysis.
- Total caspase-3 activity was measured by ELISA technique.
- Western blots were carried out in nucleus, mitochondrial, cytosol samples for the protein expression of cytochrome-c, active caspase-3 and caspase-9, PARP.
- Immunofluorescence of PARP expression in nucleus and immunolocalization of proteins cytochrome-c, caspase-3, caspase-9 and PARP was done in the small intestine segments duodenum, jejunum, ileum sections by immunohistochemistry method.
- Index of appotosis was assessed by DNA fragmentation assay.

STUDY - V was done to investigate the protective effect aminoguanidine, a selective inhibitor of iNOS on methotrexate induced enteritis.

For this study, adult male wistar rats (200 – 250g) were treated as follows:

Group I: Control rats receive vehicle alone

Group II: Rats received MTX 7mg/kg body weight intraperitoneally for 3 consecutive days.

Group III: Rats received aminoguanidine 30mg/kg body weight intraperitoneally for 3 consecutive days.

Group IV: Rats were pretreated with aminoguanidine 30mg/kg body weight 1 hour before the treatment of MTX 7mg/kg body weight intraperitoneally for three consecutive days.

Group V: Rats received aminoguanidine 50mg/kg body weight intraperitoneally for 3 consecutive days.

Group VI: Rats were pretreated with aminoguanidine 50mg/kg body weight 1 hour before the treatment of MTX 7mg/kg body weight intraperitoneally for three consecutive days.

Twenty four hours after the final dose of MTX, the rats were sacrificed and the entire length of small intestine tissue was removed, washed with saline and a piece of segments (duodenum, jejunum, ileum) was stored for histology, immunohistochemistry and immunofluorescence studies. The mucosa scrapings was collected and used for biochemical, western blot and RT-PCR analysis.

- Light microscopy of small intestine segments (duodenum, jejunum, ileum)
- Quantification of nitric oxide levels (nitrite and nitrate)
- Measurement of nitrosative stress parameters in the small intestine segments iNOS, and nitrotyrosine by IHC.
- Assay of enzyme activity of aconitase, creatine kinase, glutamate dehydrogenase, superoxide dismutase, catalase, glutathione-s-transferase, carbonic anhydrase, succinate dehydrogenase, cytochrome-c oxidase, F1-ATPase.
- NF-κB signaling pathway:
    - mRNA gene expressions of TNF-α, NFκB, iNOS, COX-2 by RT-PCR technique
    - mRNA gene expression of HO-1, HSP70, MMP2, MMP9 by RT-PCR method
    - Protein expression NFκB, COX2, PLA2 by IHC
    - Immunofluorescence of NFκB expression
- Apoptosis signaling pathway:
    - mRNA expressions caspase-3 by RT-PCR method
    - Immunolocalisation of protein cytochrome-c, caspase-3, caspase-9, parp by IHC
    - Immunofluorescence of PARP expression
    - DNA fragmentation assay

CHAPTER – 5

Materials and Methods

5.1 MATERIALS

1 – chloro – 2,4 – dinitrobenzene (CDNB), 2- theonyl trifluoroacetone (TTFA), 2'7'-dichlorodihydrofluorescin diacetate, 2-mercaptoethanol, 3-(4,5-dimethylthiazol-2-yll)-2,5-diphenyltetrazolium bromide (MTT), Alpha ketoglutarate, Aminoguanidine, Bathocuproine sulphaphonate di sodium salt (BCS), Bovine serum albumin (BSA), Chloroform, Dichlorophenol indophenol (DCPIP), Dihydrorhodamine 123, Dithiothreitol (DTT), Ethidium bromide, Ethylene diamine tetraacetic acid (EDTA), Ethylene glycol tetra acetic acid (EGTA), Follin's reagent, Glucose-6-phosphate dehydrogenase, Hexokinase, Isocitrate dehydrogenase, Isopropanol, Lactate dehydrogenase, Methotrexate, Nicotinamide adenine dinucleotide (oxidized and reduced) (NAD and NADH), Nicotinamide adenine dinucleotide phosphate (NADPH), N-naphthylethylene diamine, Oligomycin, Phenylmethanesulfonylfluoride (PMSF), Phosphocreatine, Phosphoenol pyruvate, p-Nitrophenyl acetate (PNPA), Poly-L-lysine (PLL), Pyruvate kinase, Reduced cytochrome C, Reduced glutathione, Rotenone, sodium dodecyl sulphate (SDS), Sulfanilamide, Triethanolamine hydrochloricacid (TAE-HCl), TRI-reagent, Tris-Hydrochloric acid (Tris-HCL), Ubiquinone-2, Xanthine, Xanthine oxidase are from Sigma-Aldrich, India.

4-(2–hydroxyethyl) piperazine (2–ethanesulfonic acid) (HEPES), Ammonium acetate, Copper sulphate, Dimethyl sulfoxide (DMSO), Disodium potassium phosphate, Formaldehyde, Glucose, Glycerol, Hydrochloric acid, Magnesium chloride, Methanol, Potassium chloride, Potassium cyanide, Potassium ferricyanide, Sodium carbonate, Sodium chloride, Sodium dihydrogen phosphate, Sodium hydroxide, Sodium potassium tartarate, Sodium succinate, Sucrose, Trisodium citrate, Triton-X-100, Tween-20, Zinc sulphate are from Sisco Research Laboratories, Mumbai, India.

Ethanol, Formic acid, Glacial acetic acid, Xylene from Qualigens fine chemicals, Mumbai, India. Copper-Cadmium alloy, DPX mountant, Haematoxylin and eosin, Halothane, and Paraffin. Agarose were purchased from Genei, Bangalore, India. Polyvinylidenedifluoride (PVDF) membrane (pore size 0.45 μm) was obtained from Millipore, India. Non-fat protein rich milk powder was obtained from Sagar, Gujarat, India. Super Sensitive Polymer/HRP/DAB kit was obtained from BioGenex, Chennai, India.

Antibodies against cytochrome-c, caspase-3, caspase-9, COX-2 and were obtained from Cell Signaling Technology, CA, USA. poly-ADP ribose polymerase (PARP), Beta-actin, iNOS, Anti-S-nitroso cysteine, GAPDH from sigma. Those for NFκB p65, Nitrotyrosine and were obtained from Santa Cruz, CA, U.S.A. MMP-2, MMP-9, TNFα, Secondary antibodies (anti-rabbit and anti-mouse IgG conjugated with horse-radish peroxidase) were obtained from Pierce Biotechnology, C.A, USA. Protease inhibitor cocktail (sigma-aldrich). Protein A/G Plus – agarose (santacruz). ECL dualvue western blotting markers (Amersham). West Dura chemiluminescent substrate for developing western blots was purchased from Thermo Scientific, C.A, USA.

Fluorescence labeled secondary antibodies Alexafluor 488 anti rabbit and Alexafluor 594 anti mouse, Invitrogen, CA, USA. 4′,6-diamidino-2-phenylindole (DAPI) from cell signaling, CA, USA. The reverse transcriptase core kit, qPCR master mix plus for SYBR Green kit and primer sequences were obtained from Eurogentec, Belgium. All chemicals used were of analytical grade.

5.2 METHODS

5.2.1 Animals

Adult Male Wistar Rats (200-250 gm) were used throughout the study. They were housed in standard rat cages (421 × 290 × 190 mm). All animals were exposed to 12 hour light-dark cycles and allowed access ad libitum to water and rat chow. The experiments done were approved by the institutional animal ethics committee (IAEC) and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

5.2.2 Animal treatment

Methotrexate was administered to the rats at the concentration of 7mg/kg body weight intraperitoneally for three consecutive days as described in the literature causing consistent intestinal injury in normal rats (21). Control animals received an equal volume of the vehicle (NaCl-NaOH) alone for three consecutive days intraperitoneally.

For intervention studies, aminoguanidine was administered intraperitoneally to the animals at a concentration of 30 or 50 mg/kg body weight 1hour prior to the dose of methotrexate (226). A batch of animals was administered with aminoguanidine alone 30 or 50 mg/kg body weight intraperitoneally.

5.2.3 Tissue procurement

The MTX treated rats and control rats were anesthetized with halothane and sacrificed by cervical dislocation. The abdomen of each rat was opened and the entire length of the small intestine was removed, washed with ice cold normal saline and used for studies. A piece of small

intestinal segment (duodenum, jejunum and ileum) was stored for histology and histochemistry studies. The intestine was cut opened along its anti-mesentric border longitudinally and mucosa was harvested by gently scraping with a glass slide from the remaining tissue and stored for the further studies.

5.2.4 Light microscopy

The small intestine segments (duodenum, jejunum, ileum) was fixed in 10% buffered formaldehyde, dehydrated in graded ethanol and embedded in paraffin. Five-micron sections were cut on a microtome, mounted on clear glass slides and stained with haematoxylin and eosin. The sections were examined by light microscopy (Leica) and evaluated by pathologist in a blinded fashion.

Mucosal injury, inflammation and hyperemia/hemorrhage was assessed and graded in a blinded manner using the histological injury scale previously defined by Chiu et al (227). Briefly, the mucosal damage was graded from 0 to 5 according to the following criteria: grade 0 – normal mucosal villi; grade 1 – development of sub epithelial Gruenhagen's space at the apex of the villus, often with capillary congestion; grade 2 – extension of the sub epithelial space with moderate lifting of the epithelial layer from the lamina propria; grade 3 – massive epithelial lifting down the sides of villi, possibly with a few denuded tips; grade 4 – denuded villi with lamina propria and dilated capillaries exposed, possibly with increased cellularity of lamina propria; grade 5 – digestion and disintegration of the lamina propria, hemorrhage and ulceration

5.2.5 Immunohistochemistry

The small intestine segments (duodenum, jejunum, ileum) was fixed in 10% neutral formalin and five micron sections on PLL coated slides were obtained from paraffin-embedded tissues. After deparaffinization, the sections were permeabilized for antigen retrieval with 0.1% Triton X-100 in Tris- buffered saline for 30min. Endogenous peroxidases were quenched by 3% hydrogen peroxide for 15min. After the buffer wash, the universal protein blocking agent was applied and incubated for 15min. Then, the respective primary antibody (1:100) was applied over the sections and incubated overnight followed by super enhancer for 30min. The bound primary antibody was detected by the addition of secondary antibody conjugated with horseradish peroxidase polymer and DAB substrate. Later the slides were counterstained with Harris Haematoxylin and mounted with DPX mountant medium. Sections were examined under bright field light microscopy (Olympus - cellsens standard software) and evaluated by pathologist.

IHC scoring: Young etal. (228)

Qualitative scoring Method

Score	1	2	3
Intensity of Staining	weak staining	moderate staining	strong staining

Quantitative Scoring Method

Score	0	1+	2+	3+	4+
Positive Cells	<10%	10-25%	25-50%	50-75%	>75%

There is a formula to get the percentage of positive cells (cells taking up stain of each protein)

Quick score (Q):

Results are scored by multiplying the percentage of positive cells (P) by the intensity (I).

Formula: Q = P x I; Maximum = 300 cells to be taken

5.2.6 Immunofluorescence

The small intestine tissue was fixed in 10% formalin and five micron section on PLL coated slides were obtained from paraffin embedded tissues. After deparaffinization and washing with distilled water and phosphate buffered saline (pH - 7.4) for 5min each, sections were incubated for epitope retrieval at 37ºC / 30min in phosphate buffered saline with 0.1% Triton-X- 100 (PBST). Non specific antibody binding was blocked by 2% BSA for 30min. Sections were incubated for 1hr at room temperature with respective primary antibodies (1:100), washed with PBST and followed by 1hr incubation with respective fluorescence conjugated antibodies (1:200) in a dark moisture chamber. Nuclei were counter stained with 4′,6-diamidino-2-phenylindole (DAPI) (229). Slides were mounted with 90% glycerol, the coverslips sealed with nailpolish and stored at 4ºC protected from light until imaging. The slides were observed under fluorescence microscopy (Leica) with excitation and detection of dyes. Fluorescence images were obtained with red or green with blue staining as an indication of nuclear protein expression.

5.2.7 Biochemical analysis

Isolation of cells and sub-cellular organelles

The mucosal scrapings were homogenized in homogenizing buffer containing 250mM sucrose, 5mM HEPES, 1mM EGTA, 0.5mg/ml BSA (pH – 7.4). The 10% homogenate prepared was centrifuged at 1000g for 10min at 4ºC to get nuclei pellet. The supernatant obtained was centrifuged at 10000g for 20min at 4ºC to get mitochondrial pellet. The supernatant obtained from 10000g was further centrifuged at 100,000g for 1hr at 4ºC. This final supernatant was taken as cytosolic fraction and the pellet obtained contains membranous fractions (230). All the

pellets were resuspended with homogenizing buffer without BSA, aliquot into multiple tubes, stored in -70ºC for biochemical assays.

(i) Aconitase

Activity of aconitase was measured as a rate of NADP reduction by isocitrate dehydrogenase (231)

Reagents

1. 50mM Tris – HCl (pH – 7.4)
2. 0.6mM $MnCl_2$
3. 5mM Trisodium citrate
4. 200µM $NADP^+$
5. Isocitrate dehydrogenase

Assay: To 1.0ml of Tris-HCl assay mixture added 50µl of sodium citrate, 20µl of $MnCl_2$, 10µl of $NADP^+$, 10µl of isocitrate dehydrogenase, and 30µl of sample. The change in absorbance was measured at 340nm. Aconitase activity was calculated with molar extinction co – efficient of $NADP^+$ is 6.22 x 10^3 at $A_{340\ nm}$ and expressed as unit/mg protein.

(ii) Creatine kinase

Creatine kinase activity was measured at the reduction rate of NADP by glucose-6-phosphate dehydrogenase. (232)

Reagents

1. 100mM Tris-HCl (pH – 7.4)
2. 0.15mM $NADP^+$

3. 3.3mM $MgCl_2$
4. 3.3mM glucose
5. 8.3mM phosphocreatine
6. 0.5mM ADP
7. 1.3mM AMP
8. 5µg Hexokinase
9. 5µg Glucose-6-phosphate dehydrogenase

Assay: To 1ml of reaction mixture containing 900µl Tris-HCl buffer, 10µl $NADP^+$, 10µl $MgCl_2$, 10µl glucose, 10µl ADP, 10µl AMP, 10µl phosphocreatine, 5µl hexokinase, 5µl glucose-6-phosphate dehydrogenase added 30µl of sample and increase in absorbance was measured at 340nm. Creatine kinase activity was calculated with molar extinction co – efficient of $NADP^+$ is 6.22×10^3 at $A_{340\ nm}$ and expressed as unit/mg protein.

(iii) Glutamate dehydrogenase

Activity of glutamate dehydrogenase was measured in the direction of α-ketoglutarate amination. (233)

Reagents

1. 90mM TEA-HCl (pH – 7.4)
2. 53mM ammonium acetate
3. 60µM NADH
4. 250µM EDTA
5. 13mM α-ketoglutarate

Assay: To 1.0 ml of buffer reaction mixture 100µl of ammonium acetate, 6µl of NADH, 5µl of EDTA, 100µl of α-ketoglutarate, and 30µl of sample was added. The change in absorbance was measured at 340nm and the glutamate dehydrogenase activity was calculated with molar extinction co – efficient of $NADP^+$ is 6.22 x 10^3 at $A_{340\ nm}$ and expressed as unit/mg protein.

(iv) Carbonic anhydrase

The carbonic anhydrase activity was measured by the method of reduction of p-Nitrophenyl acetate. (234)

Reagents

1. 15mM Tris buffer (pH – 7.6)
2. 3mM p-Nitrophenyl acetate (PNPA)

Assay: To 1.0ml of reaction mixture containing 740µl buffer and 250µl of PNPA added 10µl of sample and increase in absorbance was measured at 348nm. The activity was calculated with the millimolar extinction co – efficient of p-nitrophenol is 5.0 at $A_{348\ nm}$ and expressed as unit/mg protein.

(v) Superoxide dismutase

Superoxide dismutase was measured as described below (24)

Reagents:

1. 0.5M Sodium phosphate buffer pH 7.5.
2. 16% Triton X 100
3. 10mM EDTA
4. 1.2 mM MTT

5. 4 mM Xanthine
6. 1 IU of xanthine oxidase
7. 0.05 M Bathocuproine sulphaphonate di sodium salt (BCS)
8. Stop buffer: 1M Formate, 10% Triton X 100 and 40% HCHO pH 3.5.

Assay: The assay mixture consisted of 100µl of phosphate buffer, 10µl of BCS, 50µl of Triton X-100, 5µl of EDTA, 5µl of xanthine oxidase and 50µl of xanthine. To this finally 150 µl MTT and sample (50-150 µg protein) were added and the volume was made up to 1 ml with water. The mixture was incubated for 5 minutes at room temperature (37°C) and the reaction was terminated with the addition of 1ml of stop buffer. The color developed was read at 540nm. Amount of superoxide formed was calculated using the molar extinction coefficient of MTT formazan E_{540} of 17,000 M^{-1} cm^{-1} at pH 7.4 - 10.5. The percentage of inhibition of MTT color formation by the presence of SOD was calculated from the reduction of the MTT color formation as compared to the MTT formazan formed in the absence of SOD, which is taken as 100 %. One unit of SOD was defined as the amount of protein required to inhibit MTT reduction by 50%.

(vi) Catalase

Catalase activity was estimated by measuring the change in absorption at 240 nm using H_2O_2 as substrate. (24)

Reagents*:*

1. 0.05 M Potassium phosphate buffer pH 7.0
2. 30 mM H_2O_2 in 0.05 M phosphate buffer pH 7.0

Assay: To 1 ml of 30 mM buffered H_2O_2, the enzyme (sample) was added to start the reaction. The final volume was made up to 2 ml with 0.05 M phosphate buffer pH 7.0. Change in

absorbance was observed for 3 minutes at 240 nm. One unit is defined as the activity of the enzyme that disproportionates H_2O_2 at the rate of 10^{-3} absorbance/sec.

(vii) Glutathione-S-transferase

The activity of GSTase was measured spectrophotometrically using the substrate 1-chloro-2,4-dinitrobenzene (CDNB). (24)

Reagents:

1. 1 M Potassium phosphate buffer pH 6.5

2. 10 mM reduced glutathione (GSH)

3. 20 mM 1-chloro-2,4-dinitrobenzene (CDNB)

Assay: To 0.1 ml of 1M potassium phosphate buffer pH 6.5, 0.1 ml of 10 mM GSH, 0.05 ml 20 mM CDNB were added and the final volume was made upto 1 ml with distilled water. The reaction was started by adding the enzyme and change in OD at 340 nm was measured for 3 minutes. One unit of enzyme is the amount required to conjugate 1 μmole of substrate with glutathione in one minute.

(viii) Glutathione peroxidase:

Glutathione peroxidase was determined by following the oxidation of NADPH at 340 nm using hydrogen peroxide (24)

Reagents:

1. 0.4 M Sodium phosphate buffer pH 7.0

2. 4 mM EDTA

3. 10 mM Sodium azide (NaN_3)

4. 1.6 mM NADPH

5. 10mM GSH

6. Glutathione reductase (~30 units/ml of 3.6 M $(NH_4)_2SO_4$ suspension)

7. 2.5 mM Hydrogen peroxide

Assay: To 0.25 ml of 0.4 M phosphate buffer, 0.2 ml of 4 mM EDTA, 0.2 ml of 10 mM GSH, 0.2 ml of NaN_3, 0.2 ml of 1.6 mM NADPH, 0.03 ml glutathione reductase (one unit) and the enzyme (sample) were added. The total volume was made upto 2 ml with distilled water. Reaction was started by adding 0.2 ml of H_2O_2 and change in OD at 340 nm spectrophotometricallywas followed for 3 minutes. Extinction coefficient of 6.1 mm^{-1} is used for the calculation. One unit is the amount of enzyme needed to oxidize 1 nmole of NADPH/min.

(ix) Succinate dehydrogenase

Ubiquinone absorbs at 275nm but turbidity of mitochondrial and cell samples in UV range often causes problems. So, a secondary dye, Dichlorophenol indophenol (DCPIP) that absorbs at 600nm is used. Enzyme activity is that which is >90% inhibited by 2- theonyl trifluoroacetone (TTFA). (23)

Reagents:

1. Potassium phosphate buffer (100mM; pH 7.4)
2. 0.5 M Sodium succinate
3. 2.5 mM EDTA
4. 3mM DCPIP
5. 100mM KCN
6. 1mM Rotenone

7. 5mM Ubiquinone – 2
8. 100mM TTFA

Assay: The assay mixture consisted of 500µl of buffer, 40 µl of succinate, 40 µl of EDTA, 40 µl of DCPIP, 10 µl of KCN, 10 µl of rotenone and the sample in a total volume of 1ml. The reaction mixture was incubated in the spectrophotometer for 10 minutes to minimize the succinate dependent non – linear rate, and then the reaction was started by adding 10 µl of ubiquinone – 2 (or enough so that the final concentration is 50 µM). The reaction was monitored for 5minutes and then 10 µl of TTFA was added and monitored further for 5 minutes.The complex II activity was expressed as µmoles/min/mg protein using the molar extinction coefficient for DCPIP of 21mmol/L^{-1} cm^{-1}.

(x) Cytochrome – C oxidase

The oxidation of reduced cytochromec was followed at 550nm. (23)

Reagents:

1. Potassium phosphate buffer (100mM; pH 7.0)
2. Potassium ferricyanide (100mM)
3. Reduced cytochrome c

Assay: In the reference cuvette 30 µl of reduced cytochrome c, 10 µl of ferricyanide were added and the volume was made to 1ml with buffer and set to zero. In the sample cuvette 30 µl of reduced cytochrome-c and 10 µl of sample were added and the readings were monitored for 3 – 4 minutes. The enzyme activity is expressed as k/min/mg protein.

(xi) F1 ATPase

The hydrolysis of ATP by the ATPase in the mitochondrial fraction liberates ADP which is converted back to ATP by pyruvate kinase utilizing the added phosphoenolpyruvate as a substrate and energy phosphate group donor. This maintains a constant concentration of ATP and a low steady state concentration of ADP (which is an inhibitor of ATP hydrolysis). The pyruvate produced in the pyruvate kinase reaction is then converted to lactate by lactate dehydrogenase and this is coupled to the oxidation of NADH to NAD. It is the decrease in NADH at 340nm which is measured. (23)

Reagents:

1. Master mix: 100mM Tris buffer (pH 8.0), 250mM $MgCl_2$, 1M KCl, 100 mM KCN, 1mM rotenone, 5mg/ml BSA, pyruvate kinase (4 units), lactate dehydrogenase (4 units), 100 mM Phosphoenol pyruvate, 15 mM NADH.
2. 100 mM ATP
3. Oligomycin

Assay: 205 µl of master mix, 720 µl of distilled water; 5 µl of sample and 50 µl of 100mM ATP were added to the cuvette, mixed and incubated for 5 minutes. The readings were monitored for 5 minutes at 340nm and 30^0C. 20 µl of oligomycin was added and the readings were monitored for 5 min. The inhibitor sensitive rate was calculated by subtracting the rate before oligomycin addition from that observed after oligomycin addition and related this to the mitochondrial protein in the cuvette. The extinction coefficient for NADH is 6.22. The enzyme activity is expressed as moles/min/mg protein.

(xii) Nitric oxide levels

The total nitric oxide level was measured by analysis of its metabolites nitrite and nitrate levels. The combined values of nitrate and nitrite levels gives the levels of nitric oxide present in the sample.

(a) Nitrite levels

The nitrite levels are measured by using copper-cadmium alloy and griess reagent (25)

Reagents:

1. 50mM Carbonate buffer; pH 9.0
2. 3N HCl
3. 120mM $ZnSO_4$
4. 0.35M NaOH
5. Copper – Cadmium alloy
6. Griess reagent

Assay: To 100µl of homogenate, 400µl of Carbonate buffer and 0.15g of cadmium filings were added and incubated at room temperature for 1 hour with thorough shaking. The reaction was stopped by adding 100µl of 0.35M NaOH and 120mM $ZnSO_4$ and incubated at room temperature for 10 minutes and centrifuged at 4000 g for 10 minutes. 100µl of clear supernatant was aliquoted into microtitre plate wells and 50µl of 1% sulfanilamide and 50µl of 0.1% N-naphthylethylene diamine were added. The contents were mixed well and the color was read at 545nm after 10 minutes.

(b) Nitrate levels

The nitrate levels are measured by griess reagent (25)

Reagents:

1. 50mM Carbonate buffer; pH 9.0

2. 3N HCl

3. Griess reagent

Assay: To 100µl of homogenate, 400µl of Carbonate buffer, 50µl of 1% sulfanilamide and 50µl of 0.1% N-naphthylethylene diamine were added. The contents were mixed well and the color was read at 545nm after 10 minutes.

(xiii) Protein (Lowry's method)

Proteins present in the sample react with follin's reagent in an alkaline medium and the amount of blue colour developed is directly proportional to protein concentration. (235)

Reagents

1. BSA standards
2. Lowry's reagent (1% copper sulphate, 2% sodium potassium tartarate, 20% sodium carbonate in 1N NaOH)
3. Follin's reagent

Assay: The protein concentration in the sample was determined in duplicates using lowry's and follins reagent using bovine serum albumin (BSA) as standard and the intensity of blue colour developed was measured at 660nm. The concentration was expressed in mg / ml.

5.2.8 Immunoprecipitation

Small intestine mucosa samples of control and MTX treated rats was used for this experiment. Mucosal scrapings were homogenized using detergent free lysis buffer PBS (pH – 7.4) containing 5mM EDTA and protease inhibitors. The homogenates was centrifuged at 1000g for 10min to remove cell debris and to get clear supernatant and the cell fractions like nucleus, mitochondria, microsomes and cytosol were isolated using gradient centrifugation protocol. The protein concentration was measured by lowry's method as described before. An aliquot of samples 500 µg protein of cell lysate or cell fraction was used to precipitate the specific protein of interest. The samples were incubated with respective primary antibody 1 µg for 16h at 4°C. Immune complexes will be precipitated from the samples with 10 µl of protein A/G Plus – agarose (santacruz) by rotating the suspension for 4hr at 4°C. After centrifugation 2500rpm for 5min at 4ºC the beads conjugated with immune complexes were washed twice with lysis buffer. The beads will be finally resuspended in 50µl sample loading buffer containing SDS and 2-mercaptoethanol, boiled for 95ºC for 10min, electrophoresed through 10% SDS-PAGE and transferred to Millipore immobilon-P membrane for blotting.

Western blots were carried out using respective primary and secondary antibodies conjugated with horse radish peroxidase. The membrane was blocked with 5% nonfat dried milk for 1hr and incubated with primary nitrotyrosine antibody (1:1000 dilution) for overnight. The membrane was washed to remove excess primary antibody and further incubated with the secondary antibody (1:2000 dilution) conjugated to horseradish peroxidase. The membrane was then observed by supersignal chemiluminescence (pierce) and the bands were captured using AlphaEase FC gel documentation system (Alpha Innotech Corporation, CA.) and quantitated using alpha infotech fluorochem SP software. (236)

5.2.9 Western blot

(i) Purification of proteins

The mucosal scrapings was homogenized using lysis buffer containing 250mM sucrose, 20mM HEPES, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, (pH – 7.5) and added 1mM Dithiothreitol, 1mM PMSF, protease inhibitor cocktail on day of experiment. The 10% homogenate prepared were centrifuged at 1000g for 10min at 4ºC to get nuclei pellet. The supernatant obtained was centrifuged at 10000g for 20min at 4ºC to get mitochondrial pellet. The supernatant obtained from 10000g was further centrifuged at 100,000g for 1hr at 4ºC. This final supernatant was taken as cytosolic fraction and the pellet obtained contains membranous fractions. All the pellets were resuspended with homogenizing buffer, aliquot into multiple tubes, stored in -70ºC for later use. Protein concentration was determined by lowry's method and aliquots used for western blots.

(ii) Blotting

Samples containing 50-100 µg protein were denatured and separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), along with ECL DualVue western blotting molecular weight markers (Amersham). Proteins were transferred to 0.45µm polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk or bovine serum albumin (BSA) in TBS and 0.1% Tween-20 for 1hr. Thereafter, the membranes were incubated over-night in a cold room with antibodies specific to each protein of interest. After incubation with the respective primary antibodies, anti-mouse or anti-rabbit secondary antibodies conjugated with horse-radish peroxidase were used to detect bound primary antibodies. Visualization of the bands of interest was carried out by use of a chemiluminescent

substrate. The developed bands were visualized and documented using the AlphaEase FC gel documentation system (Alpha Innotech Corporation, CA.)

5.2.10 Caspase-3 activity assay by ELISA technique

Mucosal scrapings were homogenized using cell lysis buffer, kept on ice for 10 min. Centrifuged for 10,000g for 1 min in a microcentrifuge and the clear supernatant of cytosolic extract was used for the assay. Protein concentration was assessed by lowry's method. Dilute 200 μg of protein to 50 μl of cell lysis buffer for the assay in microtitre plate. Added 50 μl of 2X reaction buffer with 10 mM DTT to each sample. Added 5 μl of 4mM DEVD-pNA substrate with final concentration of 200 μM and incubate at 37ºC for 1 hour. Reading was measured at 405nm in a microtitre plate reader. The absorbance readings were used for the calculation of total caspase-3 activity.

5.2.11 Reverse Transcriptase - Polymerase chain reaction

(i) RNA isolation

Total RNA was isolated from the snap-frozen small intestinal mucosal scrapings obtained from experimental animals, using TRI-reagent. Briefly, 100 mg of mucosal scraping was homogenized in 1 ml of TRI-reagent and allowed to stand for 5 minutes at room temperature. Then 0.2 ml of chloroform was added, the contents mixed and allowed to stand for 15 minutes at room temperature. The mixture was then centrifuged at 12,000g for 15 minutes at 4ºC. The mixture separated into three phases. The colorless upper aqueous phase containing RNA was transferred to a fresh tube. Isopropanol (0.5ml) was added and the contents mixed and allowed to stand for 10 minutes at room temperature. The mixture was then centrifuged at 12,000g for 10 minutes at

4ºC. The RNA precipitate formed a pellet at the bottom of the tube. This pellet was washed by adding 1 ml of 75% ethanol. After removal of the ethanol, the pellet was air-dried for 5-10 minutes. The final RNA pellet was dissolved in 30 µl of RNAse-free water by warming the tube at 60ºC for 15 minutes. The concentration of the RNA (µg/ml) in each sample was quantified by nanodrop spectrophotometry, using nucleic acid option in nanodrop 2000/2000c software at the wavelength of 260nm. The integrity of the isolated RNA was checked by subjecting an aliquot of the sample to denaturing agarose gel electrophoresis and staining with ethidium bromide (EtBr). The integrity of the 28S and 18S subunits was used to confirm that the RNA isolated was intact and of good quality.

(ii) Synthesis of cDNA

RNA obtained was reverse-transcribed to cDNA. This was done using 1 µg of total RNA, using a reverse transcriptase core kit, according to the manufacturer's instructions (Eurogentec, Belgium). Briefly, 10 µl of reaction mixture that contained 1 µg of RNA, 1 µl of 10X reaction buffer, 2 µl of 25 mM $MgCl_2$, 2 µl of 2.5 mM dNTP, 0.5 µl of random nonamers, 0.2 µl of RNAse inhibitor, 0.025 µl of reverse transcriptase and 3.05 µl of RNase-free water were added to a microtube and mixed thoroughly.

The PCR cycler used for the conversion (Bio-Rad DNA Engine with Chromo 4 Detector System) was programmed as follows:

Initial step for 10 min at 25ºC

Reverse transcription step for 30 mins at 48ºC

Inactivation of the RT enzyme 5 mins at 95ºC

The resultant cDNA obtained was diluted to a final volume of 40 µl with RNase-free water and stored at -70ºC till use.

(iii) Real-time PCR assays

Levels of expression of mRNA were quantitated using the qPCR MasterMix Plus for SYBR green I dNTP kit (Eurogentec, Belgium), according to the manufacturer's instructions. Briefly, 25 µl of reaction mixture was prepared by taking 12.5 µl of 2X reaction buffer, 3 µl of cDNA, 5 µl each of forward (25 nM) and reverse primers (25 nM) and 4.5 µl of nuclease-free water in a PCR reaction tube. The contents of the tubes were mixed thoroughly.

The conditions for each assay were standardized with regard to annealing temperature and primer and salt concentrations. For each gene of interest, the annealing temperature used was determined by running a gradient PCR using varying temperatures. The optimum concentrations of forward and reverse primers and $MgCl_2$ were also determined.

The PCR cycler was programmed as follows for the amplification of cDNA obtained:

HotGoldStar activation step for 10 mins at 95ºC

Denaturation for 30 seconds at 94ºC

Annealing temperatures for 30 seconds

Extension for 40 seconds at 72ºC for 40 cycles.

Standard curves were generated for each gene of interest.

At the end of the amplifications, specificity of the product formed in each assay was confirmed by a melting curve analysis. The possibilities of formation of primer-dimers and non-specific amplification were thus excluded. The expression of each gene was calculated using ΔΔct method. Quantitative measurements of each gene were derived from a standard curve constructed from known concentrations of PCR product. The expression of each gene of interest was calculated relative to that of beta-actin, which was used as a house keeping gene.

Primer sequences used for PCR were given below:

iNOS	Forward	:	5'- AGG-TGT-TCA-GCG-TGC-TCC-AC -3'
	Reverse	:	5'- AGT-TCA-GCT-TGG-CGG-CCA-CC -3'
NFkB	Forward	:	5'- TGT-CCA-TGC-AGC-TTC-GGC-GG -3'
	Reverse	:	5'- GGC-CGG-GGT-TCA-GTT-GGT-CC -3'
TNF	Forward	:	5'- AAA-TGG-GCT-CCC-TCT-CAT-CAG-TT -3'
	Reverse	:	5'- TCT-GCT-TGG-TGG-TTT-GCT-ACG-AC -3'
COX2	Forward	:	5'- ATG-ACT-TCC-CTG-GGT-TTG-GT -3'
	Reverse	:	5'- GTC-CCC-CAT-TGT-GGT-ATC-TG -3'
MMP2	Forward	:	5'- ACC-GTC-GCC-CAT-CAT-CAA -3'
	Reverse	:	5'- TTG-CAC-TGC-CAA-CTC-TTT-GTC-T -3'
MMP9	Forward	:	5'- TCG-AAG-GCG-ACC-TCA-AGT-G -3'
	Reverse	:	5'- TTC-GGT-GTA-GCT-TTG-GAT-CCA -3'
HO1	Forward	:	5'- AGA-GTC-CCT-CAC-AGA-CAG-AGT-TT -3'
	Reverse	:	5'- CCT-GCA-GAG-AGA-AGG-CTA-CAT-GA -3'
HSP70	Forward	:	5'- CCG-CCT-ACT-TCA-ACG-ACT-C -3'
	Reverse	:	5'- TCT-TGA-ACT-CCT-CCA-CGA-AG -3'
CASP3	Forward	:	5'- AAT-TCA-AGG-GAC-GGG-TCA-TG -3'
	Reverse	:	5'- GCT-TGT-GCG-CGT-ACA-GTT-TC -3'
βactin	Forward	:	5'- CCT-CTA-TGC-CAA-CAC-AGT-GC -3'
	Reverse	:	5'- ACA-TCT-GCT-GGA-AGG-TGG-AC -3'

5.2.12 DNA fragmentation assay

DNA from mucosal scrapings was processed immediately after the RNA isolation protocol using TRI-reagent (Sigma, USA). Briefly, DNA was precipitated by 300 µl of absolute ethanol was added and incubate for 30 min. Centrifuged at 2000g for 5 min at 4ºC. The pellet was collected and washed thrice with 0.1M sodium citrate solution in 10% ethanol. Then the DNA pellet was isolated by centrifugation at 2000g for 5 min at 4ºC after each wash. The purified DNA precipitate formed a pellet at the bottom of the tube. This pellet was washed by adding 1 ml of 75% ethanol. After removal of the ethanol, the pellet was air-dried for 5-10 minutes. The final DNA pellet was dissolved in 50 µl of 8mM sodium hydroxide.

Isolated DNA was separated in 1% agarose gel that contained 0.05% ethidium bromide, using Tris-aceticacid-EDTA (TAE) buffer 40mM Tris base; Glacial acetic acid; 0.5M EDTA, Disodium Salt, Dihydrate; Final pH 8.0 . The separated bands were visualized and documented using an AlphaEase FC gel documentation system (Alpha Innotech Corporation, CA.) (237)

STATISTICAL ANALYSIS

Data were analyzed using the **Statistical Package for the Social Sciences (SPSS) software package, version 16.**

All parameters were studied in a minimum of 6 animals for statistical validity. The data represent the mean value ± SD and analyzed by Mann-Whitney test. Mean of multiple groups were compared by ANOVA. The data obtained were analyzed by analysis of variance (ANOVA), using a Bonferroni correction for multiple t-test as a post-hoc test. A value of $P < 0.05$ was taken to indicate statistical significance.

CHAPTER – 6

Results, Analysis and Discussion

STUDY – I

Effect of MTX on peroxynitrite (PON) - induced protein tyrosine nitration, subcellular distribution of nitrated proteins, and nitration of individual proteins in the small intestines of rats

STUDY I: Effect of MTX on peroxynitrite (PON) - induced protein tyrosine nitration, subcellular distribution of nitrated proteins, and nitration of individual proteins in the small intestines of rats

6.1.1. ABSTRACT

Methotrexate a widely used drug in clinical practice causes adverse effects on the gastrointestinal tract. These effects have been attributed to structural changes in the small intestine segments and the mechanisms such as oxidative stress and nitrosative stress play important role as shown by earlier studies. The present chapter is designed to investigate in detail the role of nitrosative stress in MTX induced mucositis. In order to carry out the study, small intestinal injury was induced in the rats by the administration of 3 consecutive i.p. injections of 7 mg/kg body weight MTX. Twenty four hours after the final dose of MTX, rats were sacrificed and the entire length of small intestine tissue was removed, washed with saline and used for histochemistry studies. Mucosal scrapings was collected and used to assess nitric oxide levels (nitrite and nitrate levels). Protein expression of iNOS and nitrotyrosine was measured by immunohistochemistry method and western blot, and iNOS mRNA was quantified by RT PCR. We found 2 fold increased nitric oxide levels, iNOS protein and mRNA expression and intense staining for iNOS and nitrotyrosine in small intestine of MTX treated rats when compared to control.

We also investigated the 3 NT in the different subcellular organelles fractions such as nucleus, mitochondria, microsomes and cytosol isolated from homogenate of mucosal samples of MTX treated rats. Nitrotyrosine western blots were carried out in all fractions and coomassie staining was done simultaneously for equal loading. Molecular weight of the bands identified was

approximately detected using molecular weight marker and the intensity of each band was calculated. After MTX treatment, a general increase in protein nitration is observed as well as a change in the spectrum of proteins that are nitrated. Comparison of the relative densities of the 3 nitrotyrosine protein adducts in the mitochondria, cytosol, nucleus from MTX treatment groups indicated that the small intestines from the MTX treated rats had greater amounts of nitrotyrosine protein adducts.

Western blot analysis showed that the bulk of the nitrated proteins had apparent molecular mass region between 12 and 72 kDa. In the nuclear fraction some proteins were more nitrated and some less compared with that of control intestine; increased nitration of 17kda, 35kda, and 58kDa proteins was observed while the proteins with molecular weights 63, 47, 40, 25, 21 showed decreased nitration after MTX treatment. In the mitochondrial fraction, many proteins were nitrated, the predominant nitrated proteins had molecular weights of 12 kda, 25kDa 29Kda, 47 kDa, and 62Kda. In the cytosol also, many proteins were nitrated. The predominant nitrated proteins had approximate molecular weights of 12kDa, 19kDa, 45 kDa, and 60 kDa. The precise localization of nitrated proteins in distinct subcellular sites such as the mitochondria, nucleus, microsomes, and cytosol implies that specific proteins are targeted for nitration by MTX.

These results suggest that MTX induced PON mediated small intestinal injury is mediated by differential nitration of proteins in the subcellular orgaenelles and selective nitration of proteins. MTX induce post translational modification of tyrosine nitration of proteins in the small intestine may play a role in MTX induced mucositis.

6.1.2. INTRODUCTION

Chemotherapy-induced alimentary mucositis is an extremely common condition that is caused by a breakdown of the mucosal barrier. It occurs in between 40 - 100% of cancer patients depending on the treatment regimen. Intestinal mucositis is a dose limiting side-effect of cancer chemotherapy, which leads to decreased absorption of nutrients, increased epithelial permeability, recurrent diarrhea, and weight loss (238). Symptoms typically include pain from oral ulceration, vomiting and diarrhoea. GIT mucositis often necessitates chemotherapy reductions or treatment breaks, overall potentially compromising survival outcomes. Consequently, it creates a burden not only on patients' quality of life but also on healthcare costs. Despite this, currently, there is no clinically effective localized/pharmacological therapy intervention strategy to prevent alimentary mucositis.

MTX is a well-known cause of intestinal mucositis, which impairs rapidly dividing cells, such as epithelial stem cells within intestinal crypts, thereby causing diminished enterocyte replacement. Generation of nitric oxide (NO) by inducible nitricoxide synthase (iNOS) is a cardinal feature of inflamed tissues including those of the gastrointestinal tract. Studies from our laboratory and others have demonstrated that nitric oxide is an important mediator of MTX induced gastrointestinal mucositis in animal models (239)

In normal conditions small amounts of NO produced by endothelial NOS (eNOS) maintains blood supply to the gastrointestinal mucosa. In inflammatory conditions, large amounts (micromolar) of NO are produced by iNOS. During inflammatory reactions when large amounts of NO and superoxide are formed, the combination of both leads to the formation of reactive

nitrogen species, such as the peroxynitrite. This toxic compound has the ability to initiate lipid peroxidation, DNA damage, sulfhydryls oxidation, and readily nitrates phenolic compounds such as tyrosine residues on proteins, resulting in augmented inflammation and tissue injury (240).

Protein 3-nitrotyrosine is a post-translational modification (PTM) of the amino acid tyrosine, with the covalent substitution of a nitrite group positioned in the 3-position in the aromatic phenol of tyrosine. Peroxynitrite induces nitration of tyrosine residues (nitrotyrosine), leading to changes of protein structure and function (107, 113). Nitration of tyrosine residues may lead to loss of protein structure and function. Protein nitration typically is indicated in diseases that have an inflammatory component, possibly caused by up-regulation of the inducible NO synthase (iNOS) (241). Varieties of post-translationally modified nitrated proteins have been shown to accumulate in apoptotic or inflamed tissues (242).

In an earlier study we have demonstrated the overproduction of reactive oxygen species, and NO, induction of iNOS and peroxynitrite overproduction has been demonstrated in the small intestines of rats after MTX treatment (25). However, the effects of PON on protein tyrosine nitration, the subcellular distribution of nitrated proteins and nitration of individual proteins has not been investigated yet to the best of our knowledge. Because peroxynitrite is impossible to measure *in vivo* owing to its high reactivity, 3-nitrotyrosine formation is used as a fingerprint of peroxynitrite formation which nitrates Tyr residues of various proteins generating 3-nitroTyr (3-NT), which is widely accepted as a foot print of peroxynitrite formation (243).

Although the participation of nitric oxide in the intestinal mucositis has been shown (68, 244), the detailed mechanism is not fully understood. As intestinal mucositis induced by MTX in a rodent model mimics the chemotherapy-associated gut damage that occurs in cancer patients; therefore, using this model, we examined the role of nitrosative stress in MTX induced enteritis.

6.1.3. AIM and OBJECTIVES

The aim of the present study is to investigate the role of PON induced protein tyrosine nitration in MTX induced enteritis. The purpose of this study was to confirm the role of nitrosative stress in MTX induced small intestinal injury.

The specific objectives were to

- Quantify the amount of nitric oxide, nitrite, nitrate in the small intestines
- Assess protein and gene expression of iNOS
- Investigate peroxynitrite production by the measurement of 3NT, footprint of PON production
- Find out whether there is any difference in the extent of PTN of proteins in the subcellular organelles of the small intestines
- Identify the molecular weights of proteins that are nitrated

6.1.4. SUMMARY OF EXPERIMENT

Adult male wistar rats (200 – 250g) were treated with MTX 7mg/kg body weight and the control rats were treated with vehicle alone intraperitoneally for three consecutive days. After 24 hours of the final dose of MTX the rats were sacrificed and the entire length of small intestine tissue

was removed, washed with saline and a piece of segments (duodenum, jejunum, ileum) was processed for histology, iNOS and nitrotyrosine histochemistry studies.

Mucosal injury, inflammation and hyperemia/hemorrhage was assessed and graded in a blinded manner using the histological injury scale previously defined by Chiu et al. (227). The mucosa scrapings was collected, and used for measuring nitrate, nitrite biochemical assay, iNOS gene expression by RT-PCR, nitration of proteins by immunoprecipitation and western blots. Mucosa scraping was also used for isolation of cellular fractions (nucleus, mitochondria, microsomes, cytosol) from the homogenate. SDS-PAGE technique followed by western blot of nitrotyrosine was done using monoclonal anti-nitrotyrosine antibody in all the cell fractions. Coomassie brilliant blue staining was done in all the fractions as a proof for proteins bands in the samples as well as to check degradation of proteins and equal loading of the sample. The molecular mass of each bands identified in the blots was calculated based molecular weight marker. Band intensity was quantitated for each bands identified in the blots. Difference in the band intensity between control and methotrexate treated samples indicates the increase or decrease in nitration of the respective protein band.

6.1.5. RESULT

MTX administration resulted in moderate to massive injury to the small intestines.

Under light microscope, the duodenum, jejunum and ileum of the control group rats showed normal architecture (Figure: 6.1.1 A – C). The mucosa was lined by villi which are short and leaf like in duodenum, tall in jejunum and of intermediate height in ileum. The villus epithelium consisted of an admixture of tall columnar epithelial cells and goblet cells, which are more numerous in ileum. Crypts of lieberkuhn formed the lower 20% of the epithelium and were surrounded by per crypt fibroblast sheath. Lamina propria was composed of loose connective tissue matrix containing lymphocytes, and plasma cells. Eosinophils, macrophages and neutrophils were occasionally seen along with blood vessels, nerves and lymph vessels.

Twenty four hours after the final dose of MTX, there was moderate destruction of the villi and the crypts of leiberkuhn in duodenum (Figure: 6.1.2 A). In jejunum, there was more destruction of the villi. The villi were atrophied and focally absent (Figure: 6.1.2 B). In the ileum, the villi were atrophied, blunted and fused. There was destruction of the crypts of leiberkuhn (Figure: 6.1.2 C). The villus/crypt ratio was decreased and there was transmural acute inflammatory infiltrate in the mucosa, submucosa and muscularis layers. The overall thickness of the epithelium was decreased accompanied by increased intraepithelial lymphocytes, neutrophil infiltration and crypt abscess.

Based on the light microscopic changes the intestine sections from the two groups were graded on a 5 point scale previously defined by Chiu et al (227). The small intestine sections in control group revealed normal morphology with normal villi and crypt cells (Grade 1). The duodenum

and jejunum sections in MTX group showed atrophied villi with exposed lamina propria and there was acute inflammatory infiltrate (Grade 4). The ileum sections from the MTX group revealed fused villi, digestion and disintegration of the lamina propria in villi and presence of hemorrhage and ulceration (Grade 5). The damage was comparatively more in the ileum (grade 5), followed by jejunum and duodenum (grade 4).

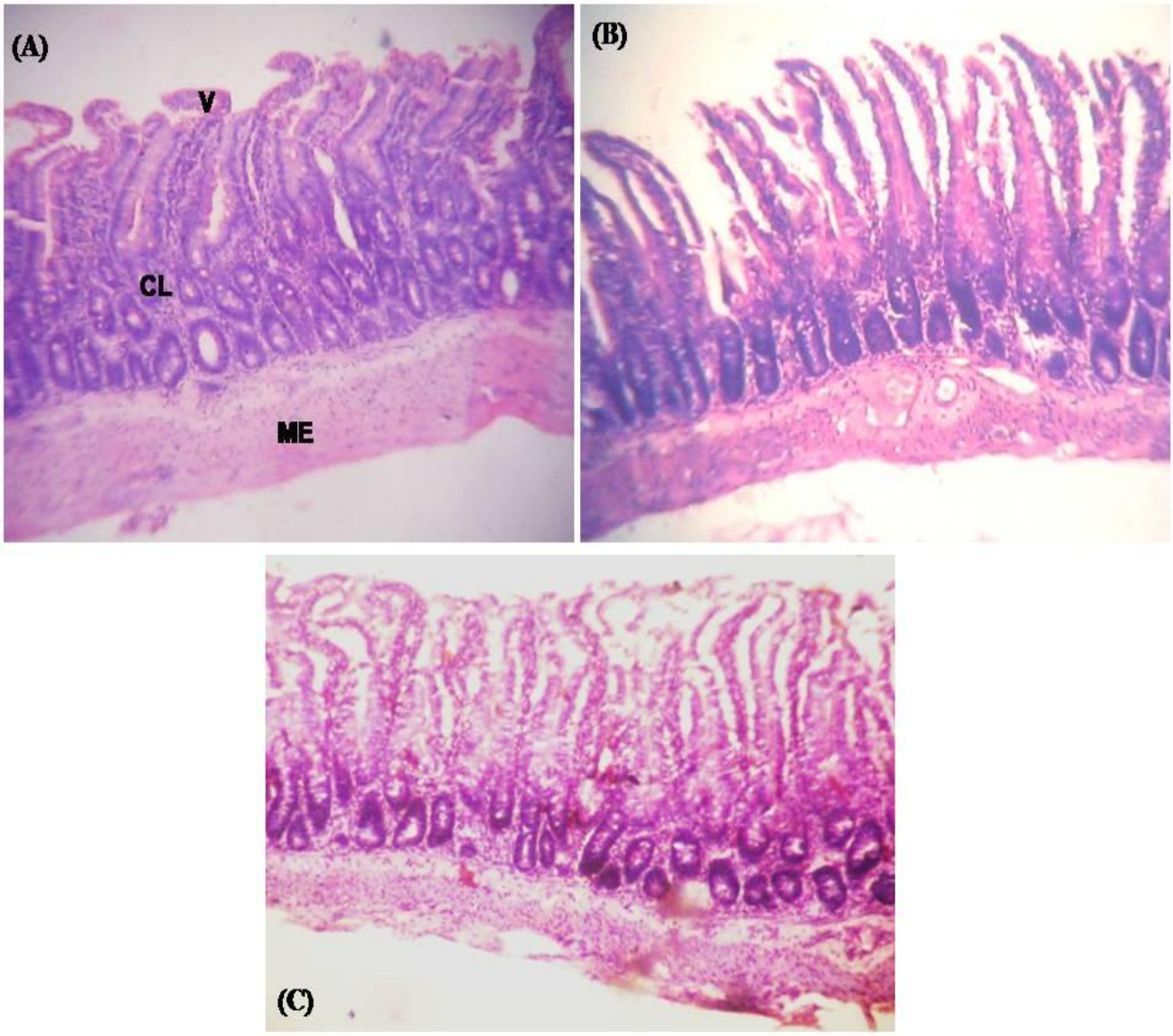


Figure: 6.1.1

Histology of the duodenum (A), jejunum (B) and ileum (C) of control rats showing normal architecture, magnification X100. (n = 6).V – Villus, CL – Crypts of Leiberkhun; ME – Muscularis Externa

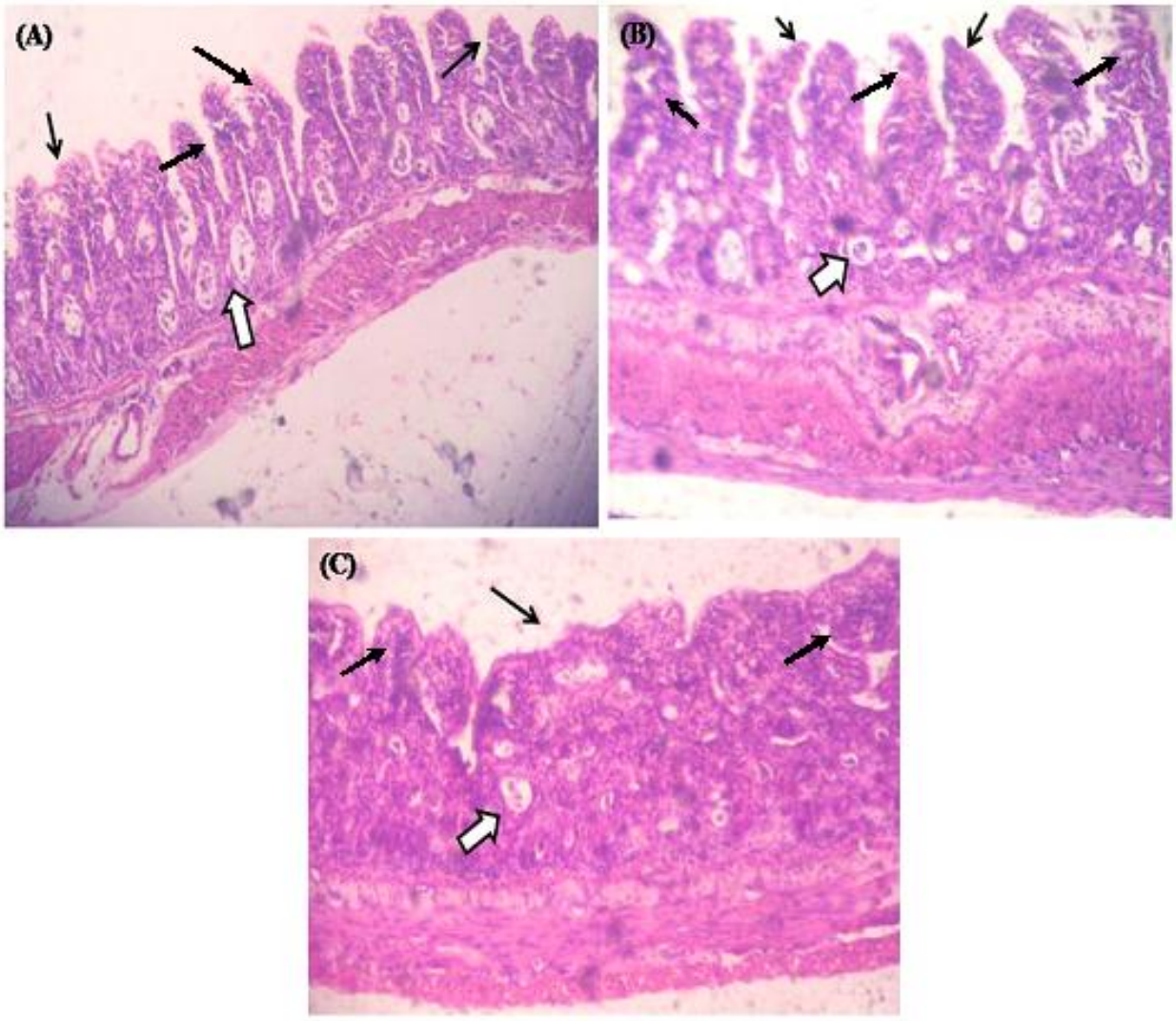


Figure: 6.1.2

Histology of small intestine 24 hours after the final dose of MTX, magnification X100. (n = 8). Black arrow indicates the apoptotic villi and the white arrow indicates the crypt abscess. The villi were shortened in the duodenum (A), distorted in the jejunum (B) and aborted, flattened, blunted and fused in the ileum (C).

MTX administration resulted in more than 2 fold increase in the levels of NO (nitrite and nitrate) in the small intestines

Nitrate and nitrite levels were measured by using copper cadmium alloy and griess reagent spectrophotometric method in the small intestine mucosa of vehicle and MTX treated rats. Nitrate + nitrite levels were calculated as an index of nitric oxide production. The small intestines of control rats showed low basal levels of nitric oxide. This suggests the physiological role of NO in the small intestines. The source of this NO may be the expression of constitutive NOSes - eNOS and nNOS, and to a lesser extent iNOS. MTX treatment resulted in significant increase in the levels of nitrate, nitrite and thus nitric oxide as compared to control. Nitrite levels were increased by 2.1fold (14.08 ± 3.4 vs. 6.82 ± 1.82, P = 0.002), nitrate levels by 2.4 fold (41.65 ± 6.3 vs. 17.31 ± 3.48, P = 0.002) and nitric oxide by 2.3 fold (55.73 ± 19.5 vs. 24.12 ± 7.4, P = 0.002) in MTX as compared with control (Figure: 6.1.3). The source of this NO may be upregulation of iNOS

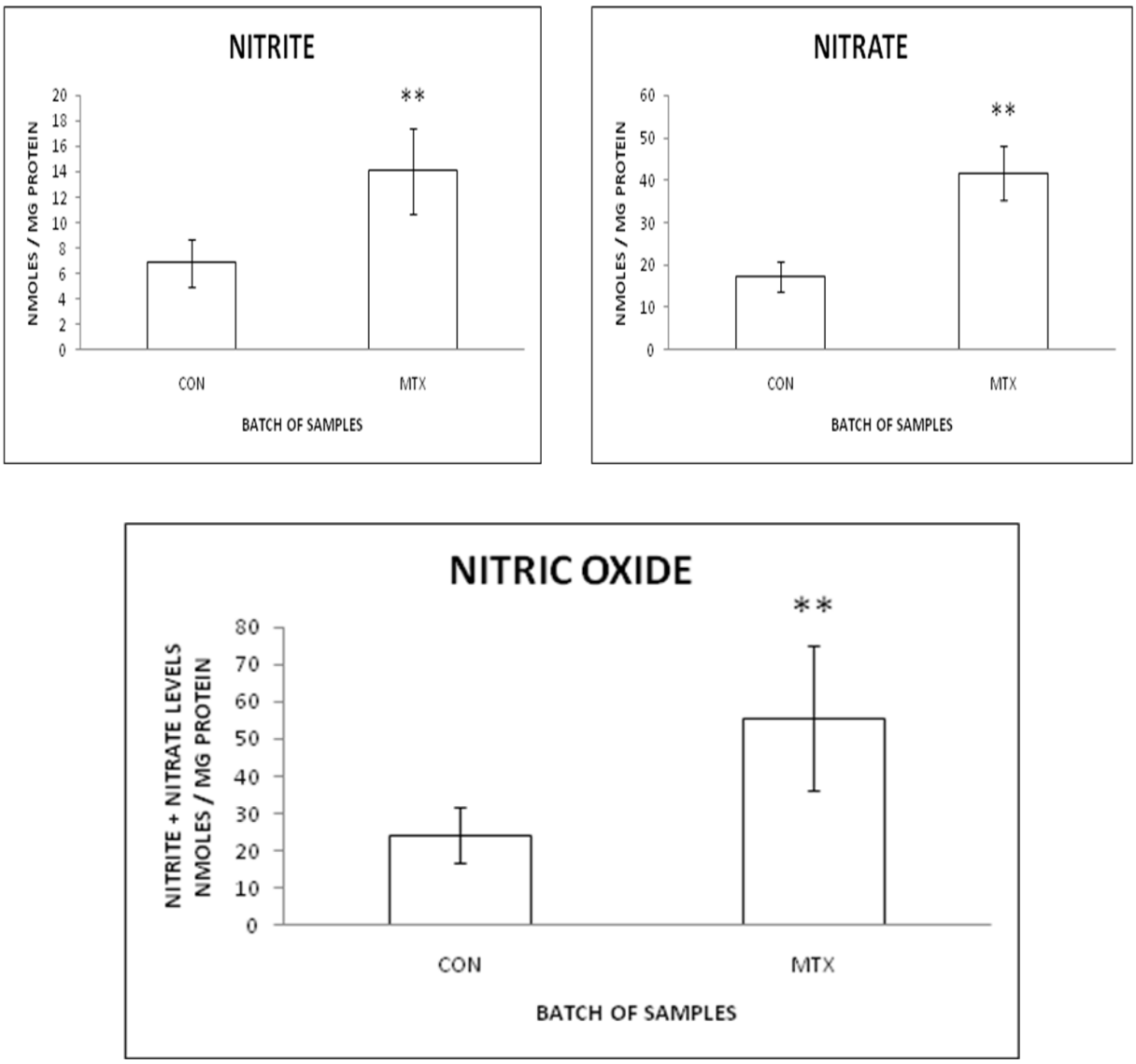


Figure: 6.1.3

Nitrite, Nitrate and Nitric oxide levels in the small intestine mucosa of control and experimental rats, 24 hours after the final dose of vehicle and MTX respectively. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control.

MTX treatment resulted in increased iNOS protein and mRNA expression

Immunohistochemical analysis of the control rat intestines showed weak immunostaining for iNOS in the villus and crypt regions. iNOS is only expressed in some inflammatory cells scattered throughout the lamina propria. MTX-treated rats presented intense iNOS immunostaining in the enterocytes, lamina propria cells, and neutrophils and other inflammatory cells surrounding and within necrotic crypts when compared to the weak immunostaining of villus and crypt regions from unchallenged rats (Figure: 6.1.4). iNOS protein localized primarily to the epithelial cells lining the crypts and along the crypt axis and abundant in the villus, suggesting that intestinal epithelial cells may be the prime source of the gut mucosa-derived NO and contribute to the increased levels of NO2/NO3 in the lumen of the small intestine after MTX challenge. These results suggest that inducible NOS is constitutively expressed in enterocytes, and is increased in rat intestines after MTX treatment.

The effect of MTX on iNOS transcription and iNOS mRNA accumulation was confirmed by reverse transcriptase polymerase chain reaction (Figure: 6.1.5). Untreated rat intestines contained detectable amount of iNOS mRNA suggesting its basal expression. MTX treatment resulted in more than 2 fold increase (0.0076 ± 0.003 vs 0.0036 ± 0.0001, $P = 0.029$) iNOS mRNA as compared to control. These findings strongly suggest role for iNOS in MTX induced small intestinal Injury.

iNOS IHC

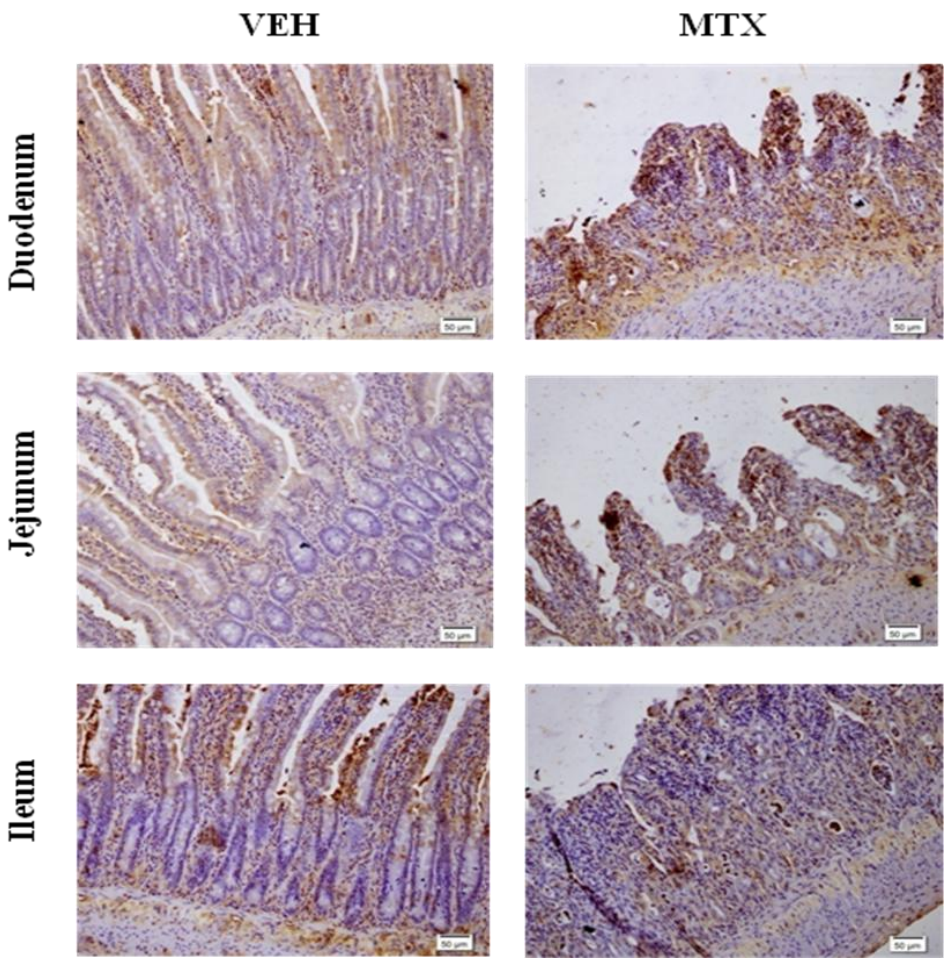


Figure: 6.1.4 - Representative image of inducible nitric oxide synthase (iNOS) protein expression in the small intestine segments of rats 24 hours after the treatment with vehicle and MTX by immunohistochemical analysis (20X). MTX treated rats shown intense immunostaining of iNOS in the duodenum, jejunum and ileum when compared to basal immunostaining in control rat small intestine tissue treated with vehicle.

Site	Duodenum	Jejunum	Illeum
VEH	+/-	+/-	+
MTX	++	++	+++

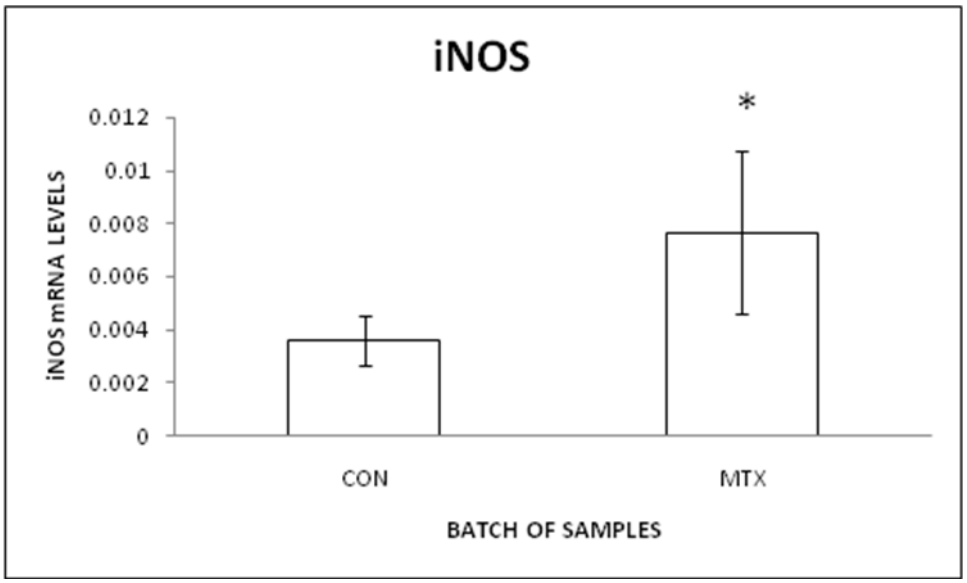


Figure: 6.1.5 - Effect of MTX on iNOS mRNA levels. RT-PCR analysis of iNOS mRNA expression after 24hours of final dose of MTX and vehicle treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

MTX administration resulted in more than 2 fold increase in protein tyrosine nitration in the small intestine

Protein tyrosine nitration, is indicative of nitric oxide-driven peroxynitrite and/or free-radical damage. Therefore, we next assessed the level of tyrosine-nitrated proteins generated by MTX by immunohistochemistry as an indication of reactive nitrogen products. Intestines of control rats showed weak staining for 3NT. Animals treated with MTX showed intense staining for nitrotyrosine residues, both in enterocytes and lamina propria cells, in contrast to the weak immunostaining seen in the control intestines (Figure: 6.1.6).

In order to confirm the formation of 3NT adducts, we carried out western blots in whole intestinal homogenates and probed with NT antibody. 3 Nitrotyrosine adducts were present in detectable amounts in the small intestines of control rats. The small intestinal homogenates from all of the MTX treated rats had higher nitrotyrosine protein adducts as compared with the controls. On an average increase in 3NT was observed in the MTX treated rat intestines 1.1 fold (1512.7 ± 134.8 vs. 786.6 ± 523.8, $P = 0.20$) (Figure: 6.1.7). These results clearly demonstrated that there was some intrinsic nitration in some proteins in the normal intestine, and the intrinsic protein nitration was significantly enhanced after MTX injury.

NTY IHC

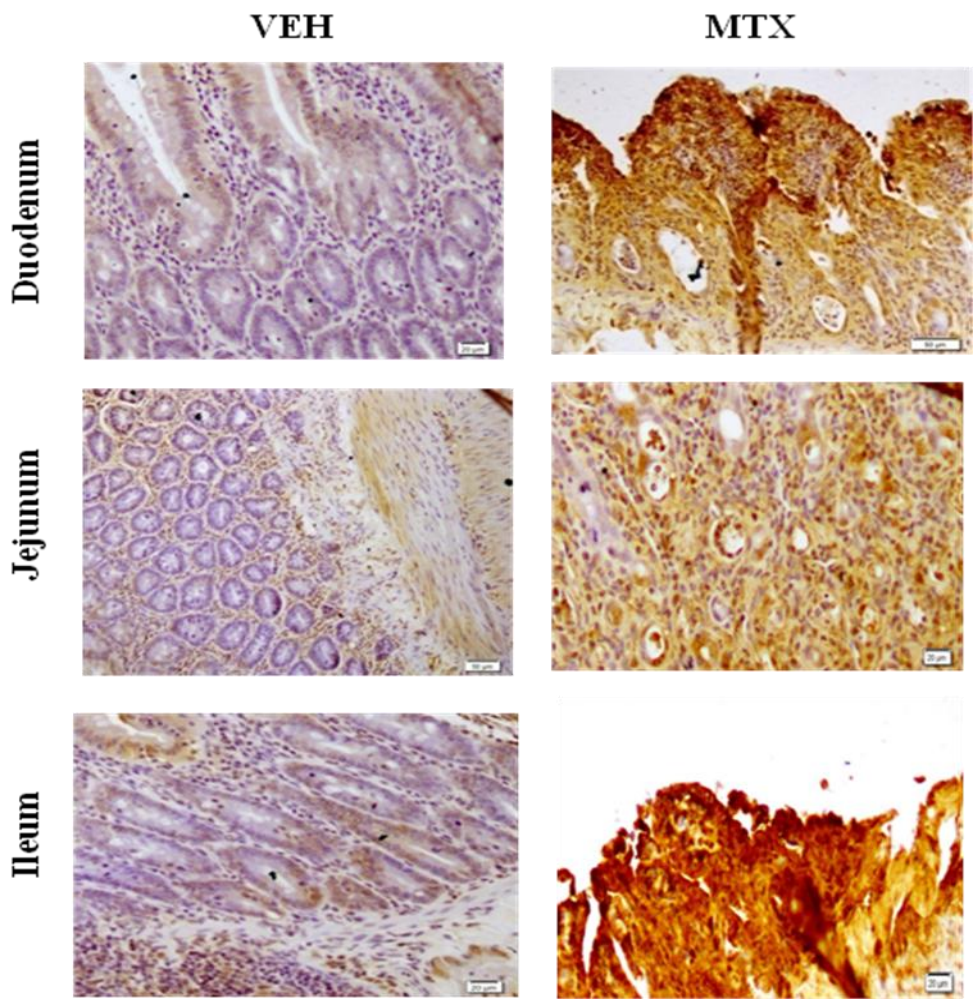


Figure: 6.1.6 – Representative image of nitrotyrosine (NTY) protein expression in the small intestine segments of rats 24 hours after the treatment with vehicle and MTX by immunohistochemical analysis (20X). MTX treated rats shown intense immunostaining of NTY in the ileum followed by duodenum and jejunum when compared to basal immunostaining in control rat small intestine tissue treated with vehicle.

Site	Duodenum	Jejunum	Illeum
VEH	+/-	+/-	+
MTX	++	++	+++

MTX results in differential nitration of subcellular proteins

After confirming the overproduction of 3NT in the MTX treated rat intestines, we next investigated whether there is any difference in the extent of protein nitration in the various subcellular compartments of the enterocytes. To directly compare the levels of nitrated proteins in the subcellular organelles between the control and the MTX treated small intestines, the subcellular organelle fractions were subjected to immunoprecipitation IP followed by WB using monoclonal anti-NT antibody. According to Guo et al., immunoprecipitation with an anti-nitrotyrosine antibody is the preferred technique for detecting unknown tyrosine-nitrated proteins in tissue (245).

For this, individual small intestines obtained from normal rats and MTX treated rats were homogenized and the subcellular organelles i.e, mitochondria, nucleus, microsomes, and cytosol were separated by differential centrifugation. The proteins in various subcellular organelles were immunoprecipitated, subjected to Western blot analysis with the monoclonal anti-NT antibody. The extent of nitration of proteins was measured by densitometric analysis. In all the subcellular organelles there was basal expression of NT. These data indicate that nitric oxide-dependent protein tyrosine nitration is a physiologically relevant process localized within specific subcellular compartments After MTX treatment, a general increase in protein nitration is observed as well as a change in the spectrum of proteins that are nitrated (Figure: 6.1.7).

Comparison of the relative densities of the 3 nitrotyrosine protein adducts in the mitochondria, cytosol, nucleus from MTX treatment groups indicated that the small intestines from the MTX treated rats had greater amounts of nitrotyrosine protein adducts as compared with control. In

contrast, there was significant decrease in nitrotyrosine adduct formation in the microsomes. The major site of PON production is the mitochondria, but nitration of proteins was observed in almost all the extra-mitochondrial fractions in addition to the mitochondria. These suggest that nitration can occur in any subcellular compartment independent of the site of peroxynitrite production.

As indicated by densitometric analysis the total protein nitration in the whole homogenates was increased to 1.1 fold (1513 ± 135 vs. 786.6 ± 523.8) after MTX treatment. Western blots using the 4 subcellular organelles, protein nitration was increased in nucleus by 1.3 fold (842.3 ± 172.7 vs. 330.7 ± 220.5), mitochondria by 0.7 fold (2371 ± 305 vs. 1448 ± 584.2), microsomes by 1.0 fold (1561 ± 308.6 vs. 763.7 ± 312.1), and cytosol by 0.5 fold (1700.7 ± 313.4 vs. 1076 ± 233.5), respectively in MTX treated as compared to that of untreated control (Figure: 6.1.7). The different magnitude of enhancement suggests that protein nitration in vivo is specific and selective.

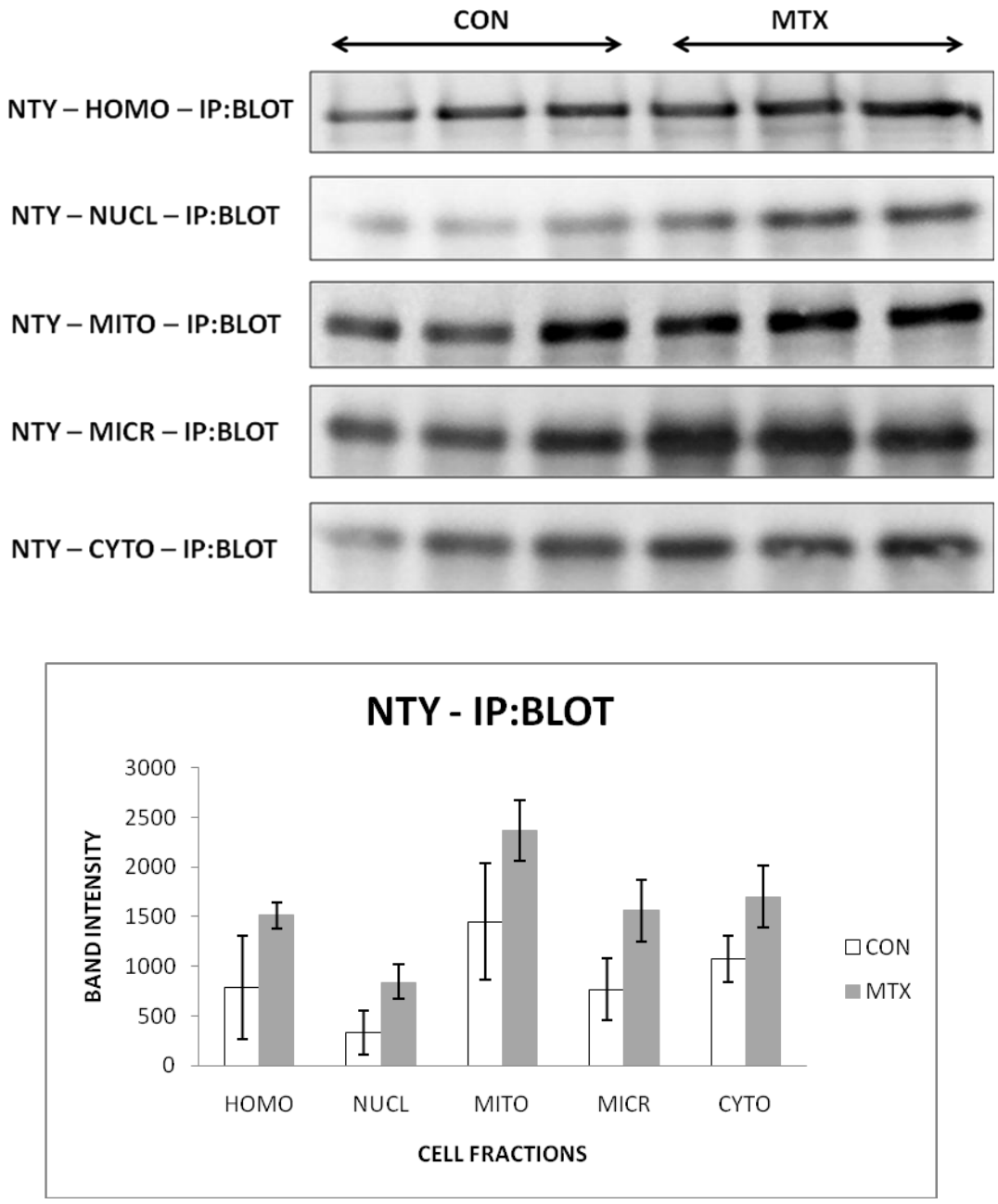


Figure: 6.1.7 – Representative blot and corresponding graph of nitrotyrosine protein levels. The overall nitrotyrosine levels were done by immunoprecipitation followed by western blot method. Nitrotyrosine levels in the small intestine mucosal cell fractions of control and experimental rats, 24 hours after the final dose of vehicle and MTX respectively. Abbreviations – Nitrotyrosine (NTY), Immunoprecipitation followed by western blot (IP:BLOT), Homogenate (HOMO), Nucleus (NUCL), Mitochondria (MITO), Microsomes (MICR), Cytosol (CYTO). Data represent mean ± SD, N = 3 in each group.

MTX targets specific subcellular proteins of the small intestines

Next, we wanted to identify the molecular weights of individual proteins that may be nitrated in the various sub cellular compartments of the small intestines upon MTX treatment. For this, proteins present in the subcellular organelles were separated by SDS-PAGE along with molecular weight markers and stained with coomassie blue to identify the protein bands present in the sample. Another set of same samples were separated by SDS-PAGE and then subjected to western blot analysis with the anti-3-NT antibody to identify the protein bands that undergo nitration.

SDS-PAGE combined with Western blot analysis followed by densitometric analysis, showed that in all the sub cellular organelles many proteins were nitrated under basal conditions, and MTX treatment increased their nitration. In control rats, all enterocyte preparations showed positive staining for nitrotyrosine. Staining was particularly marked between 12 and 70 kDa in all samples tested.

As shown in (Figure: 6.1.8), the small intestinal homogenates from all of the MTX treated rats had higher nitrotyrosine protein adducts as compared with the controls displays a representative Western blot analysis, showing the bulk of the nitrated proteins had apparent molecular mass region between 12 and 72 kDa. We got positive bands with approximate molecular masses 57 kDa (270.3 ± 181 vs. 182.3 ± 66.6), 54 kDa (331.3 ± 235.7 vs. 166.3 ±84.3), 35 kDa (357 ± 127.6 vs. 96.3 ± 54.2), 25 kDa (1081.3 ± 169.4 vs. 576 ± 153), and 21 kDa (4972 ±837.8 vs. 2482 ± 991.3) in response to methotrexate when compared to control.

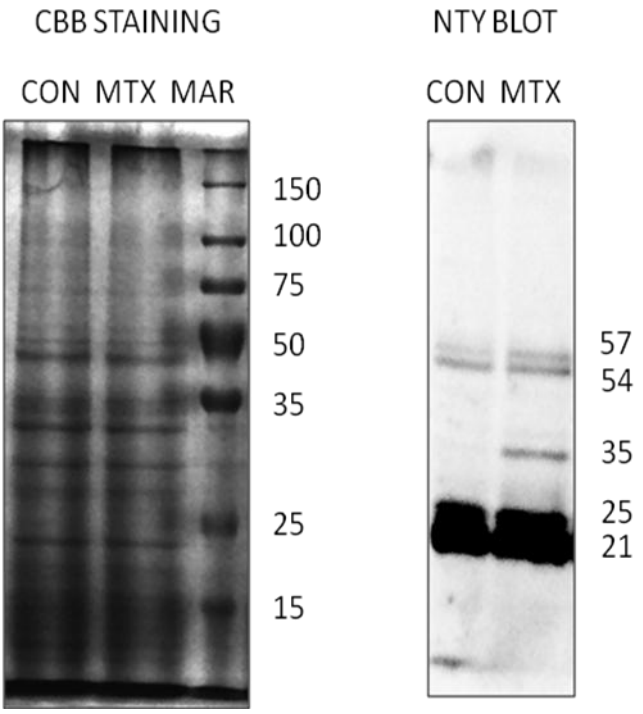
In the nuclear fraction some proteins were more nitrated and some less compared with that of control intestine; increased nitration of 17kDa (982.7 ± 192.8 vs. 330.3 ± 259.1), 35kDa (38.3 ± 3.1 vs. 12 ± 1.4), and 58kDa (176 ± 28.8 vs. 45 ± 16.5) proteins was observed while the proteins with molecular weights 63 kDa (52 ± 13.7 vs. 99.3 ± 20.2), 47 kDa (130.3 ± 51.1 vs. 423.7 ± 200.9), 40 kDa (16.3 ± 9.29 vs. 44 ± 13.5), 25 kDa (58.3 ± 18 vs. 619.6), 21 kDa (81.3 ± 26.9 vs. 499 ± 119.1) showed decreased nitration after MTX treatment as compared to control (Figure: 6.1.9).

In the mitochondrial fraction, many proteins were nitrated, the predominant nitrated proteins had molecular weights of 12 kDa (201.7 ± 153.9 vs. 79.7 ± 61.1), 21kDa (1550.3 ± 144.3 vs. 908 ± 173.3), 27kDa (83.7 ± 24.6 vs. 45 ± 14.7), 35kDa (99.7 ± 22.1 vs. 33.3 ± 20.2), 52 kDa (94.7 ± 37 vs. 40.7 ± 3.5), and 57kDa (108.3 ± 69.5 vs. 30 ± 7) were decreased nitration in MTX treated compared to control (Figure: 6.1.10).

In the microsomes, proteins with molecular weight 14 kDa (78.7 ± 15.3 vs. 333.7 ± 81.4), 16 kDa (79 ± 12.1 vs. 911.3 ± 74,), 19 kDa (104.7 ± 24.7 vs. 691.7 ± 158.2), 21 kDa (160 ± 27 vs. 918.7 ± 91.7), 22 kDa (151.3 ± 16.9 vs. 811 ± 150.1), 27 kDa (45 ± 21.4 vs. 544.3 ± 116.6), 33 kDa (146 ± 44.2 vs. 744.7 ± 54.8), 38 kDa (46.3 ± 35.6 vs. 327 ± 78.6) showed decreased nitration after MTX treatment compared to control (Figure: 6.1.11).

In the cytosol also, many proteins were nitrated. The predominant nitrated proteins had approximate molecular weights of 12kDa (916 ± 254.1 vs. 406.3 ± 77.7), 19kDa (799 ± 257.3 vs.

Figure: 6.1.8 **HOMOGENATE SAMPLES**



Coomassie brilliant blue staining, nitrotyrosine blot and representative quantification of bands identified in nitrotyrosine blot of homogenate samples of control and MTX treated small intestine mucosa samples.

Western blotting analysis of nitrotyrosine of homogenate samples revealed increased nitrated protein expression with approximate molecular masses 57 kDa, 54 kDa, 35 kDa, 25 kDa, and 21 kDa in response to methotrexate when compared to control.

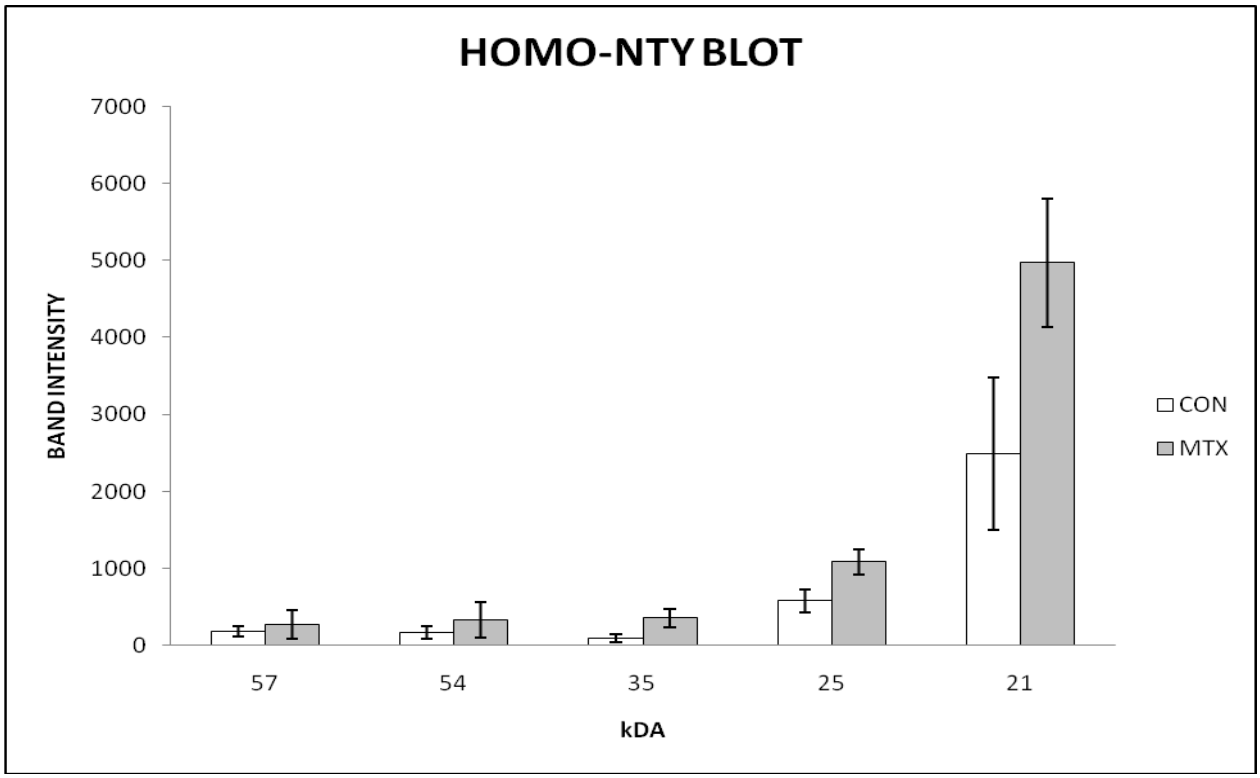
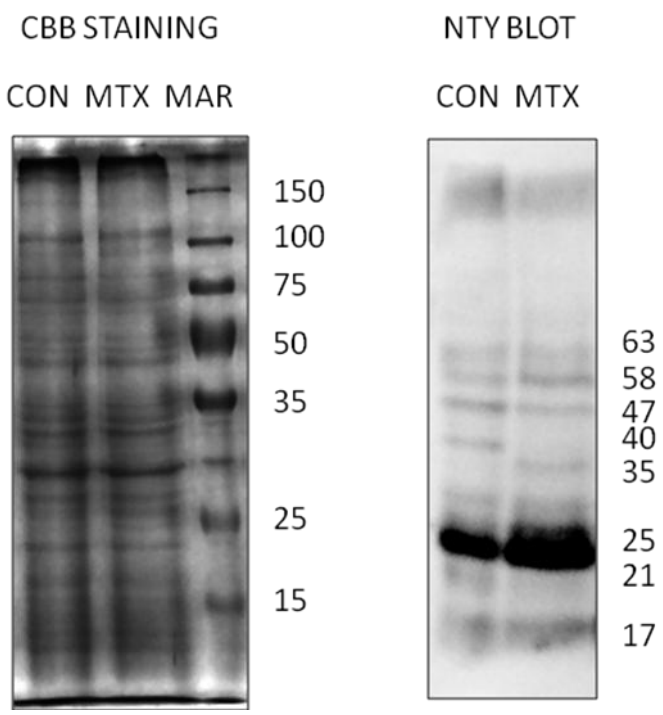


Figure: 6.1.9 **NUCLEUS SAMPLES**



Coomassie brilliant blue staining, nitrotyrosine blot and representative quantification of bands identified in nitrotyrosine blot of nucleus samples of control and MTX treated small intestine mucosa samples.

Western blotting analysis of nitrotyrosine of nucleus samples revealed increased nitrated protein expression with approximate molecular masses 58 kDa, 35 kDa, 17 kDa and decreased nitrated protein expression with approximate molecular masses 63 kDa, 47 kDa, 40 kDa, 25 kDa, 21 kDa in response to methotrexate when compared to control.

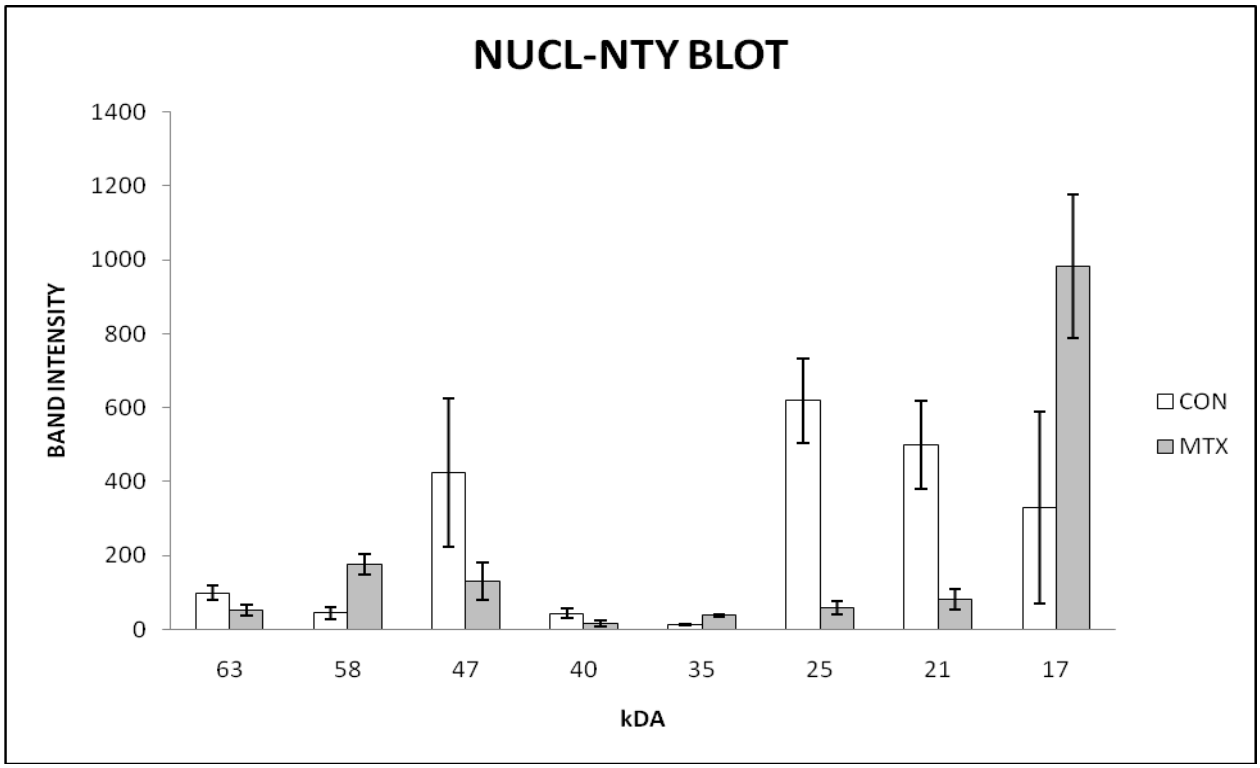
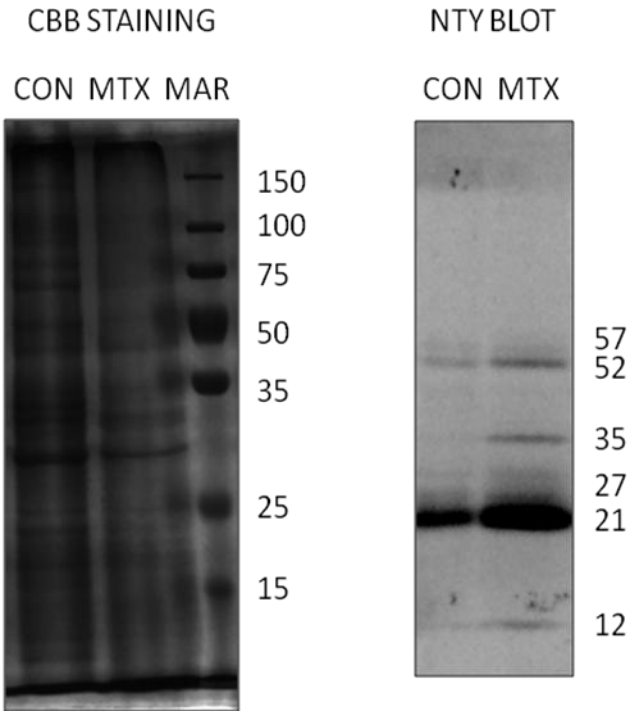


Figure: 6.1.10 **MITOCHONDRIA SAMPLES**



Coomassie brilliant blue staining, nitrotyrosine blot and representative quantification of bands identified in nitrotyrosine blot of mitochondria samples of control and MTX treated small intestine mucosa samples.

Western blotting analysis of nitrotyrosine of mitochondria samples revealed increased nitrated protein levels with approximate molecular masses 57 kDa, 52 kDa, 35 kDa, 27 kDa, 21 kDa, 12 kDa in response to methotrexate when compared to control.

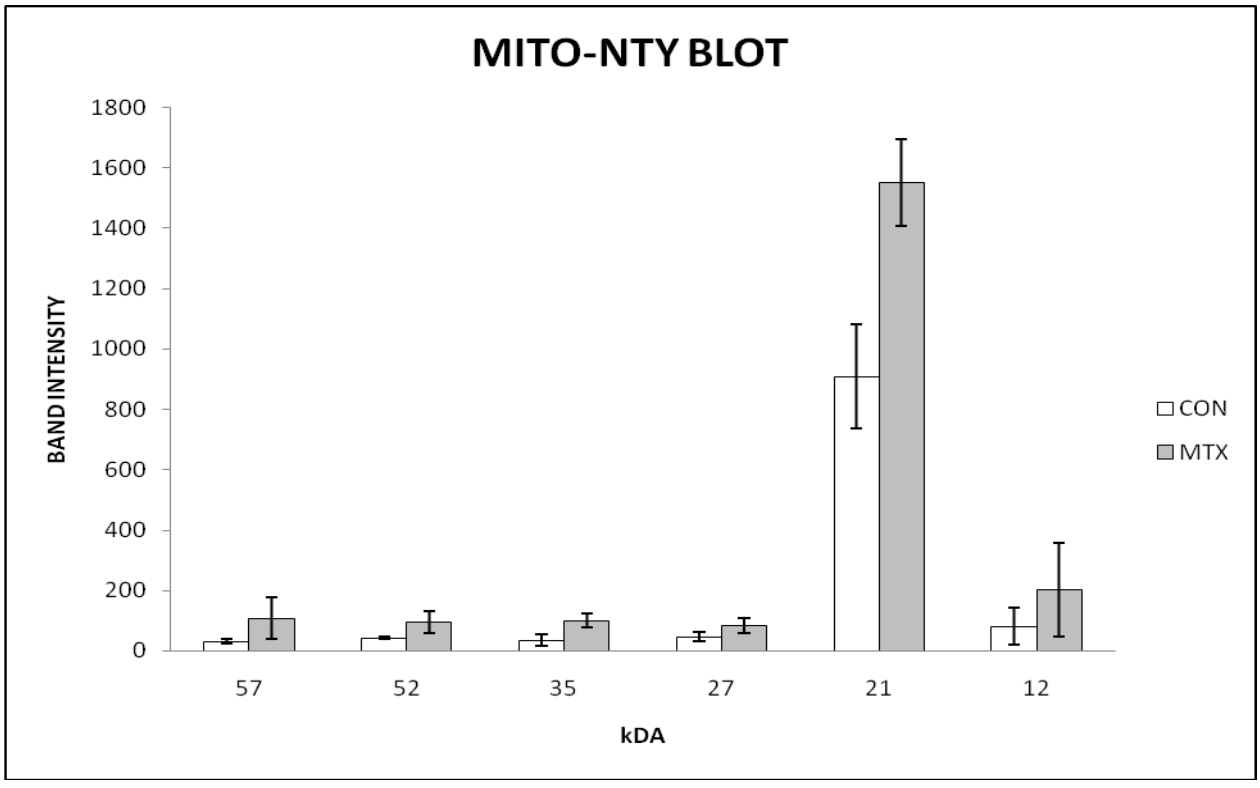
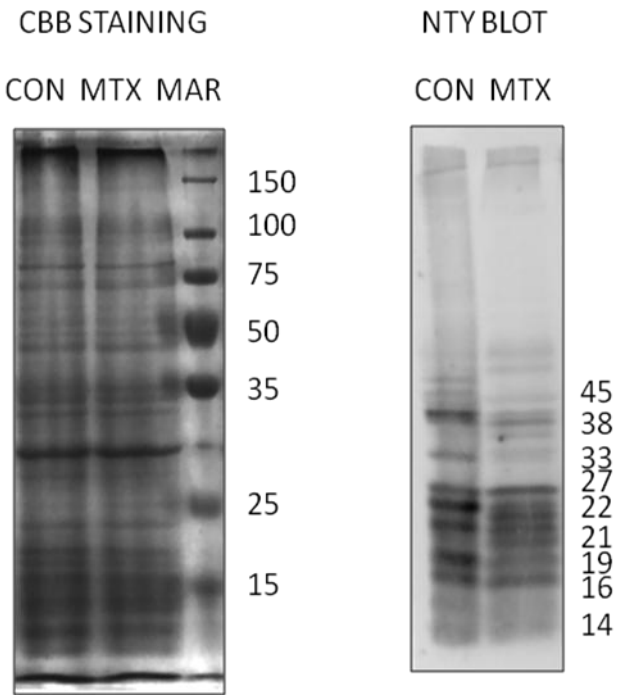


Figure: 6.1.11 **MICROSOME SAMPLES**



Coomassie brilliant blue staining, nitrotyrosine blot and representative quantification of bands identified in nitrotyrosine blot of microsome samples of control and MTX treated small intestine mucosa samples.

Western blotting analysis of nitrotyrosine of microsome samples revealed decreased expression of nitrated proteins with approximate molecular masses 38 kDa, 33 kDa, 27 kDa, 22 kDa, 21 kDa, 19 kDa, 16 kDa, 14 kDa and no change in expression with molecular mass 45 kDa in response to methotrexate when compared to control.

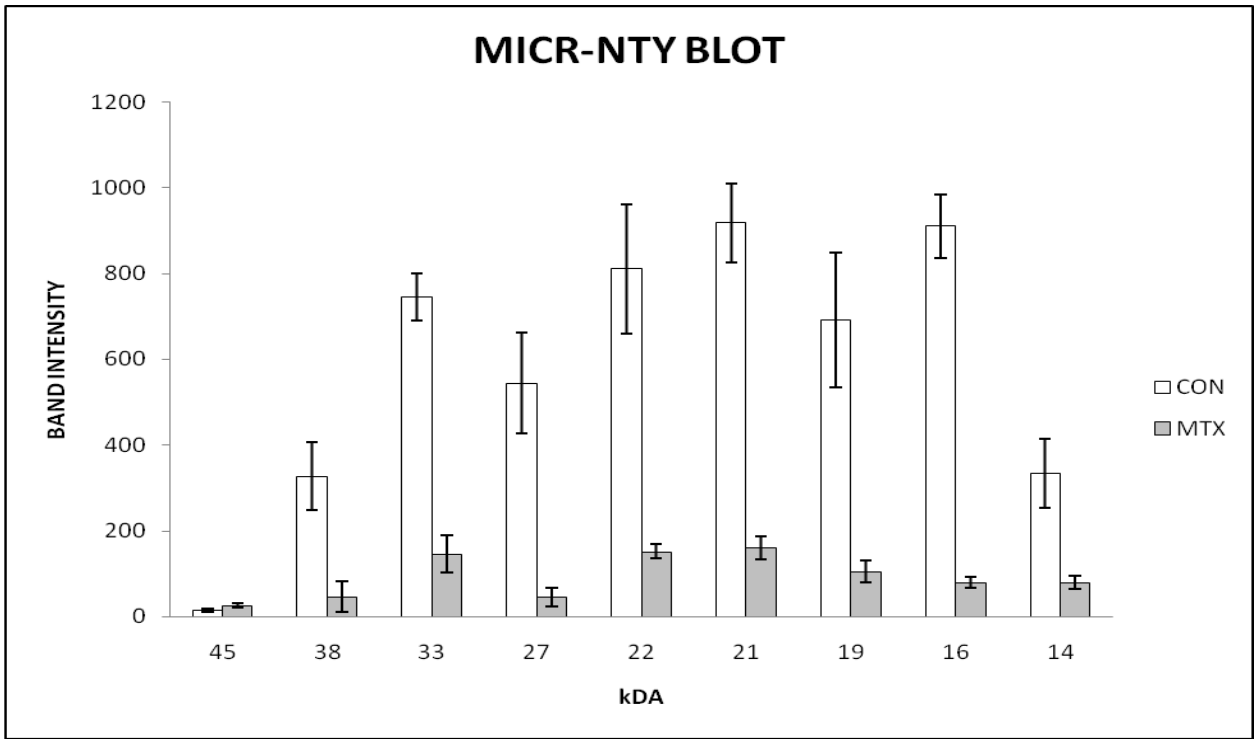
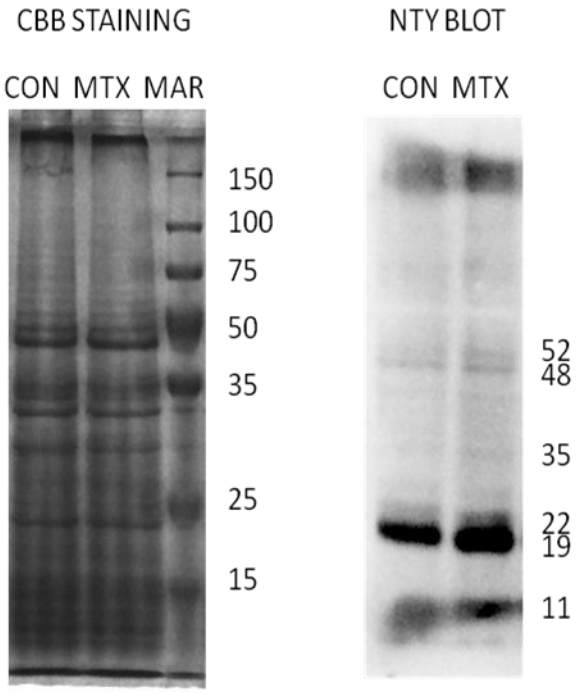
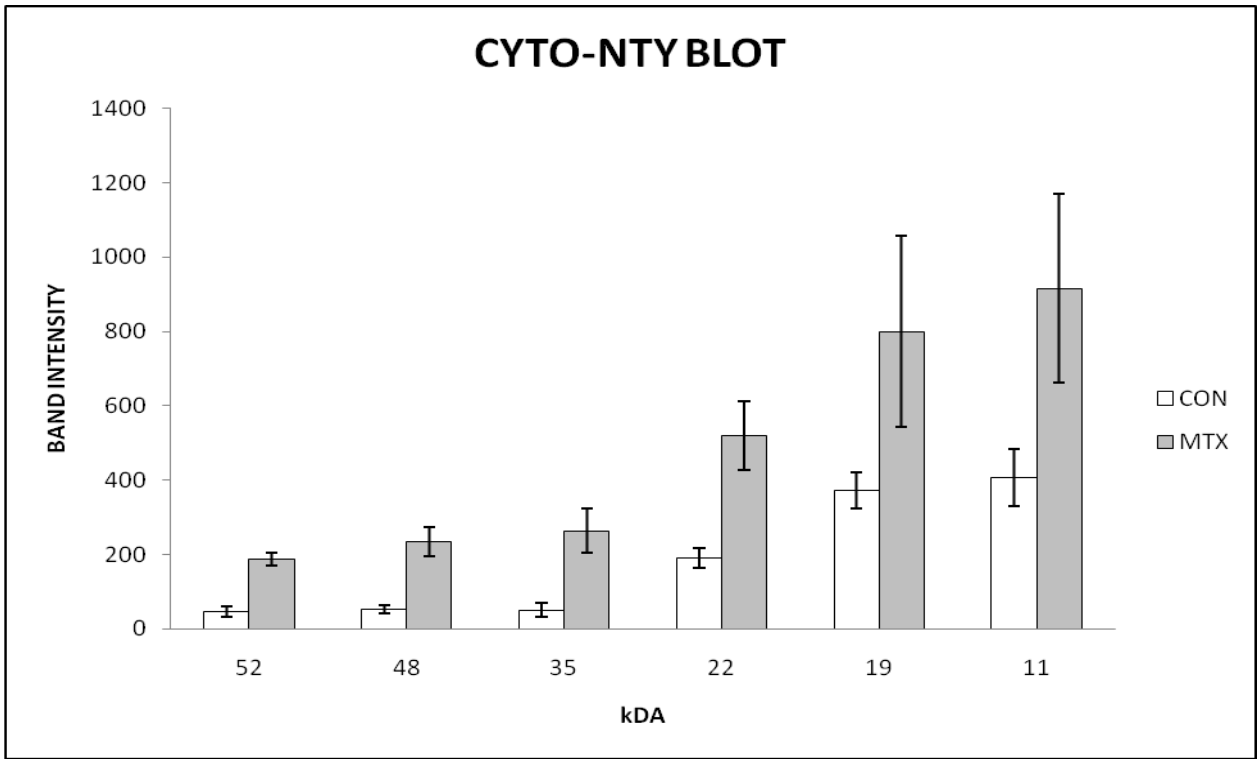


Figure: 6.1.12 **CYTOSOL SAMPLES**



Coomassie brilliant blue staining, nitrotyrosine blot and representative quantification of bands identified in nitrotyrosine blot of cytosol samples of control and MTX treated small intestine mucosa samples.

Western blotting analysis of nitrotyrosine of cytosol samples revealed increased nitrated protein levels with approximate molecular masses 52 kDa, 48 kDa, 35 kDa, 22 kDa, 19 kDa, 11 kDa in response to methotrexate when compared to control.



373 ± 48.4), 22 kDa (518.7 ± 93.1 vs. 189.7 ± 27.1, 35kDa (263.7 ± 60.4 vs. 51 ± 19), 45 kDa (234.3 ± 38.6 vs. 52.7 ± 11.2), and 60 kDa (186.7 ± 16.3 vs. 46.3 ± 13.2) (Figure: 6.1.12).

The precise localization of nitrated proteins in distinct subcellular sites such as the mitochondria, nucleus, microsomes, and cytosol implies that specific proteins are targeted for nitration by MTX. These results suggest that MTX mediated small intestinal injury is mediated by differential nitration of proteins i.e., increased nitration of selected proteins, and decreased nitration of some other proteins.

It has been demonstrated that in ‘in vivo ‘inflammatory conditions certain proteins are selectively nitrated and their activity altered. We compared the molecular mass of the proteins that are nitrated upon MTX treatment with the molecular mass of proteins that are known to undergo nitration in inflammatory states.

Based on this, we speculate that the proteins that undergo nitration in the mitochondria of MTX treated rat intestines correspond to cytochrome c molecular weights 12 kDa, MnSOD 25kDa, carbonic anhydrase 29 kDa, creatine kinase 47 kDa, and glutamate dehydrogenase 62 kDa. In the cytosol molecular weights of nitrated proteins correspond approximately to cytochrome c 12kDa, caspase 3 19kDa, GST 45 kDa, and catalase 60 kDa

MTX treatment resulted enhance nitration of aconitase, SDH. A proteomic approach toward targets of protein nitration in both *in vivo* and cell culture inflammatory disease modelsh identified more than 40 nitrotyrosine-immunopositive proteins, became modified as a

consequence of the inflammatory response. Therefore next, we attempted to identify the individual proteins that undergo nitration.

Three critical enzymes aconitase, Mn SOD, Succinate DH selected for further characetisation with respect to nitration. These proteins were enriched by IP with specific antibodies. After IP, Western blotting with the monoclonal anti-3-NT antibody (mouse IgG) was performed. For all the proteins studied, protein levels were not significantly different between control and MTX, but the extent of nitration was higher for aconitase, and SDH in the MTX treated rats as compared with control. With respect to SOD, we were not able to demonstrate significant nitration.

6.1.6. DISCUSSION

In order to investigate the role of nitrosative stress in MTX induced small intestinal damage, we first estimated the nitric oxide levels (measured as the stable degradation production, nitrites and nitrate) in the small intestinal homogenates. The control rat small intestines showed measurable amount of nitric oxide level. The basal production of NO in the small intestines of control rats indicates a physiological role for NO. In fact, nitric oxide (NO) plays a critical role in several of the physiological processes that occur in the GI tract including motility, secretion, digestion, absorption and elimination. The importance of NO in GI mucosal defense is well established (99). NO also contributes to mucosal defense through its cytotoxic properties, a primary defense against ingested bacteria and parasites (100). The actions of NO overlap considerably with those of prostaglandins: modulation of the activity of mucosal immunocytes (e.g., mast cells and macrophages), reduction of leukocyte-endothelial adhesive interactions, modulation of mucosal blood flow, reduction of epithelial permeability, stimulation of mucus, and bicarbonate secretion (246). NO has proven to be the primary non-adrenergic non-cholinergic neurotransmitter in the GI tract (101). In the present study, MTX treatment resulted in more than two fold increase in NO. High concentrations of NO are related to numerous pathological processes of GIT including peptic ulcer, chronic gastritis, gastrointestinal cancer, bacterial gastroenteritis, celiac or chronic inflammatory bowel diseases (103).

NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). The GIT expresses three types of NOS. The constitutive (calcium dependent) isoforms, neuronal NOS (nNOS or bNOS) and endothelial NOS (eNOS), produce small amounts of NO which acts as a neurotransmittor and vasodilator respectively. Recent studies indicate that iNOS is expressed in

a small amount and plays a role in intestinal mucosal integrity under physiological conditions (102). The inducible (calcium independent) isoform (iNOS) produces much larger amounts of NO and is expressed during inflammation. iNOS is induced by cytokines like interferon-gamma(IFN-γ), tumor necrosis factor α, interleukin-1 (IL-1) and lipopolysaccharide (LPS). iNOS induction is suppressed by transforming growth factor (TGF-β), and interleukin -8 and -10 (IL-8,-10). There is a general agreement that NO derived from the constitutively expressed NOS (e.g., eNOS or nNOS) is "protective/anti-inflammatory and the large amounts of NO derived from the inducible iNOS is proinflammatory. Therefore, it is not surprising to find basal amounts of NO in control rat intestines. In the present study we were able to demonstrate basal levels of iNOS protein and mRNA expression in normal rat intestines. The presence of iNOS in normal tissue of intestine should not be surprising, because recent studies show that small amounts of NO produced by iNOS has physiological role. iNOS is expressed in a small amount and plays a role in intestinal mucosal integrity under physiological conditions (102). Because the gut epithelium is constantly exposed to the foreign antigens/noxious substances entering gastrointestinal tract either with the food or produced by the bacteria residing in the intestine as part of the normal intestinal milieu (247), basal expression of iNOS may have protective effects. Under pathological conditions, excessive iNOS and NO, however, are harmful to intestinal mucosal barrier (102, 150, 248). Recently, this hypothesis that cNOS is always beneficial and iNOS is always deleterious, has been questioned, since that a series of data suggest that the increase of cNOS activity could be responsible for the derived pathological changes and, by contrast, NO liberated by the inducible isoenzyme might play a repairing effect in certain pathological disorders (103).

In the present study, MTX treatment resulted in induction of iNOS protein and mRNA expression as compared with control and was accompanied by inflammatory changes in the small intestines as evidenced by elevated MPO (shown in our earlier publication) (24) and TNF levels (shown in chapter 3). It has been shown that iNOS-derived NO can act as a homoeostatic regulator of inflammatory leucocyte recruitment and the role of iNOS in leucocyte recruitment has been examined in several inflammatory models, including intestinal inflammation (249-250). In the gut, inflammatory, epithelial, endothelial and neuronal cells can express iNOS (251). In the present study iNOS protein was detectable in the enterocytes and infiltrating leucocytes. It has been shown earlier that in inflamed gastrointestinal tissue, iNOS is richly expressed by infiltrating and resident activated macrophages (225, 252). The NO generated by activated macrophages may have significant physiological benefits. For example, NO produced by macrophage iNOS has important antimicrobial functions and inhibits the growth of viruses, parasites, and gram-positive organisms that invade the gastrointestinal tract. Although macrophage derived NO appears to be important in innate immunity, the high diffusion efficiency of NO makes it potentially reach unintended targets (e.g., neighboring epithelial cells). Inflammatory cytokines will also trigger iNOS expression by epithelial cells. Thus the salutary effects and potentially detrimental consequences of NO generation in inflammation exist along a continuum related to the magnitude and chronicity of NO exposure.

Several studies have demonstrated that intestinal epithelial cells express iNOS during inflammatory conditions such as celiac disease, inflammatory bowel disease (IBD), diverticulitis, and endotoxemia. Overproduction of NO and upregulation of iNOS have been reported to play role in intestinal inflammation caused by different agents including anti-cancer drugs, LPS, I/R

injury. Lima-Júnior R et al have shown using a rat model that overproduction of NO, upregulation of iNOS play role in pathogenesis of irinotecan-induced intestinal mucositis (253). Induction of iNOS and overproduction of NO have been reported to play a role in FU and MTX induced mucositis in rats (225, 239). Upregulation of iNOS mRNA and protein expression as well as overproduction of NO have been reported to play role in acute endotoxemia induced intestinal mucosal injury in rats (254). When stimulated by LPS, NO is excessively expressed and induces multi-organ functional lesions in stomach and intestine of mice (255). Inhibition of NO production has been shown to protect the intestines from injury (218).

NO can be directly cytotoxic but can also react with superoxide anions (O_2•-) yielding the oxidizing agent peroxynitrite (ONOO-). During inflammatory reactions when large amounts of NO and superoxide are formed, the combination of both leads to the formation of reactive nitrogen species, such as the peroxynitrite. This toxic compound has the ability to initiate lipid peroxidation, sulfhydryls oxidation, and readily nitrates phenolic compounds such as tyrosine residues on proteins, resulting in augmented inflammation and tissue injury (256-257)

The reaction between superoxide anion and NO not only yields peroxynitrite a potent pro-oxidant, but also alters levels of nitric oxide, which in turn affect physiological functions. The formation of peroxynitrite within cells and tissue from the reaction of cellular nitric oxide (NO) with the superoxide radical was first proposed by Beckman nearly two decades (258).

The peroxynitrite anion is extremely unstable thus evidence of its formation in vivo has been indirect via the occurrence of nitrated moieties including nitrated lipids and nitrotyrosine residues in proteins. Formation of 3-nitrotyrosine (protein nitration) is a “molecular fingerprint”

of peroxynitrite formation. The standard index of peroxynitrite formation in vivo is the formation of nitrated tyrosine residues. Protein nitration has been widely reported in a number of pathological states associated with inflammation but is reported to occur in normal physiology and is thought of as a prevalent, functionally relevant post-translational modification of proteins. The formation of nitrotyrosine has been observed in numerous states of chronic inflammation (243, 259)

Immunohistochemistry is an important and often used tool in the investigation of nitrotyrosine, which is a marker of oxidative injury that is frequently linked to altered protein function during inflammatory conditions (243, 260). Nitrotyrosine can be detected in multiple species, organ systems, tissues, and cell types during inflammation. Therefore we analysed 3-NT in the intestinal tissues by immunohistochemical methods and western blot. Basal levels of 3NT were detectable in the small intestines of control rats suggesting a physiological role for PTN. Protein 3-nitrotyrosine is a post-translational modification (PTM) of the amino acid tyrosine, with the covalent substitution of a nitrite group positioned in the 3-position in the aromatic phenol of tyrosine (261). The modification is mediated by reactive nitrogen species and can be formed under a number of physiological conditions. Western blots using anti-nitrotyrosine antibodies demonstrate that multiple proteins are modified under normal conditions (262-264). The role protein nitration plays in cell physiology is unclear.

A significant increase of nitrotyrosine immunostaining was observed in the intestinal segments of MTX-treated rats. In addition we were able to demonstrate using western blot technique more than 2 fold increase in 3NT adducts in MTX treated rat intestine as compared with control,

reinforcing the role of NO via peroxynitrite on intestinal mucositis. The degree of protein nitrotyrosine formation is considered an indication of the production of reactive nitrogen species and of potential cell damage (265).

Studies have shown that sustained NO production and ONOO- formation occurring in inflammatory states may differentially accelerate apoptosis in the villus apex and/or inhibit proliferation at the base of the crypts resulting in expanded extrusion zones at the villus tip resulting, at least transiently, in a "bare area" at the villus tip where bacteria can attach and traverse the epithelium. Therefore, Thus ONOO- may promote gut barrier failure not only by inducing enterocyte apoptosis but also by disrupting signaling pathways involved in enterocyte proliferation (118-119).

Tyrosine nitration has often been regarded as non-selective oxidative damage to proteins; however, recently more specific roles have been described.8 For example, tyrosine nitration triggers dissociation of IκB from Nuclear Factor-κB (NF-κB), which results in activation of this pathway that plays an important role in cancer and inflammation (266-267).

Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific means for detection of the “footprint” of peroxynitrite (111). Recent studies indicate, however, that certain other reactions can also induce tyrosine nitration. Nitrite and hydrogen peroxide may be used by peroxidases, such as myeloperoxidase, to generate nitrogen dioxide, another potent nitrating agent. It is noteworthy to mention here that we observed massive increase in MPO activity in the small intestine of MTX treated rats in our previous publication, suggesting that this may also contribute to protein tyrosine nitration (24-25).

Another possible mechanism involves the Fenton reaction. Increase in NO leads to release of heme from proteins that can then serve as catalysts for nitration of adjacent proteins. In the presence of H_2O_2, the heme is converted to ferryl π-cation radical species, which subsequently converts nitrite to $\cdot NO_2$ to increase nitration. Thus, increased nitrotyrosine staining is considered to be an indicator of "increased nitrosative stress" rather than a specific marker of peroxynitrite (243).

Peroxynitrite anion is formed mainly from the fast diffusion controlled reaction between nitric oxide and superoxide, where nitric oxide out competes SOD for superoxide. Key regulators of peroxynitrite formation are therefore amounts of NO and superoxide (109). Indeed, mitochondria are central intracellular sources of $O_2^{\cdot-}$, and peroxynitrite formation is favored by the facile diffusion of $^{\bullet}NO$ from the cytosol, main sites of PON production. Because of low levels of glutathione and antioxidant enzymes in mitochondria than in cytosol, mitochondrial lipids, DNA, and proteins can be more susceptible to oxidative and nitrative modifications under increased nitrative stress one would expect mitochondrial proteins to be nitrated preferentially (23).

To determine whether there is any preferential subcellular protein targets for PON, the small intestines were homogenized, subcellular organelles separated and their nitration status was examined by Western blotting analyses. Coomassie staining of the protein samples showed that there was no obvious difference in the intensity of the protein spots between controls and MTX treated groups. In all the subcellular organelles of the small intestines, there was basal expression of NT. These data indicate that nitric oxide-dependent protein tyrosine nitration is a

physiologically relevant process localized within specific subcellular compartments in close proximity to iNOS (268). These results clearly demonstrated that there was some intrinsic nitration in some proteins in the normal heart, and the intrinsic protein nitration was significantly enhanced after MTX injury. After MTX treatment, a general increase in protein nitration was observed as well as a change in the spectrum of proteins that are nitrated. MTX treatment resulted in nearly 2 fold increase in 3-nitrotyrosine formation in cytosol, mitochondria, nucleus and microsomes. Studies have shown that PON has a half life less than 100 ms which allows it to travel distances of 5-20 μm (1-2 cell diameters) across extra and intracellular compartments. Once formed, peroxynitrite can permeate cell membranes through either anion channels or passive diffusion of the anionic and protonated forms, respectively (269), and over a biological half-life of ~5–20 ms, it promotes toxic effects locally at up to one- to two-cell diameters . This may provide explanation for the detection of nitrated proteins in many subcellular oragelles, although mitochondria are the main site of PON production. This suggests a role for increased PTN and ubiquitous nitration of proteinds in MTX incuded enteritis

Studies carried out using different agents have shown that subcellular nitration of proteins induced by the agents is both organ specific and agent specific. Challenge of mouse dendritic cells with lipopolysaccharide induced iNOS protein expression in cytosol and peroxisomes and was associated with an increased 3-nitrotyrosine formation in cytosol, mitochondria, and peroxisomes. Organ specific and Site specific nitration of proteins have been reported in drug induced and LPS induced tissue injury (270). In AAP induced liver injury, significant increase in nitrotyrosine adduct formation in the mitochondrial and cytosol, But no significant the nucleus or in microsomes (271). Nitrotyrosine immunoreactivity, increased in the hippocampal region

after acute exposure to combustion smoke revealed predominantly mitochondrial and cytoplasmic staining.

There are many reports on nitrated proteins in various diseases, identified by current proteomic methods such as 2D-PAGE or LC-MS/MS. The biological functions and the subcellular locations of identified nitrated proteins are classified into multiple categories. Of the identified nitrated proteins in *in vivo* disease models, 25%, 20%, 28% are derived from mitochondria, extracellular, and cytoplasm or intracellular, respectively. Since, various active redox reactions occur in mitochondria, it is expected to be the center of nitration. The major nitrated proteins identified are shown to be involved in energy metabolism (20%), which includes many redox reactions (272)

Having found the difference in extent of nitration of proteins in the various subcellular compartments, we were interested in finding out the molecular weight of protein that undergo nitration in the subcellular oragnelles of the small intestines after MTX treatment.. In the mitochondrial and cytosolic fraction extensive nitration of proteins with molecular mass ranging from 12-52 KDa was observed. In the nuclear fraction some proteins were more nitrated and some less compared with that of control intestine; increased nitration of 17Kda,35Kda, and 58KDa proteins was observed while the proteins with molecular weights 63,47,40 25, 21 showed decreased nitration after MTX treatment. In the microsomes, proteins with molecular weight 14 - 38 showed decreased nitration after MTX treatment, while the overall 3NT in the microsomes was increased. The different magnitude of enhancement suggests that protein nitration induced by MTX in the small intestines is specific and selective.

Studies have shown that different agents target proteins of different molecular weights. Upon acetaminophen treatment, the liver homogenates from all of the mice had nitrotyrosine protein adducts. The predominant nitrated proteins had molecular weights of 36 kDa, 44 kDa, and 85 kDa. A protein with a molecular weight of 85 kDa appeared to have the greatest concentration of nitrotyrosine (273). I/R in an in vivo rat myocardial I/R model, marked increase in 3-nitrotyrosine formation with several major bands located at 45-50 and 25-30 kDa (274). In TCE induced the nitrated proteins spots were found in the pI range of pH 4.9 to 8.8 and molecular weights of 19.1 to 128.3 kDa (275). Aniline exposure led to increased tyrosine nitration of splenic proteins, as determined by 2D Western blotting with anti-3-nitrotyrosine specific antibody, compared to the controls. The analyzed nitrated proteins were found in the molecular weight range of 27.7 to 123.6kDa (276).

A proteomic approach toward targets of protein nitration in both *in vivo* and cell culture inflammatory disease models identified more than 40 nitrotyrosine-immunopositive proteins that became modified as a consequence of the inflammatory response. These targets include proteins involved in oxidative stress, apoptosis, ATP production, and other metabolic functions (272).
Three critical enzymes aconitase, Mn SOD, SDH were selected for further characetisation with respect to nitration post MTX treatment. According to Guo et al. (245), immunoprecipitation with an anti-nitrotyrosine ntibody is the preferred technique for detecting unknown tyrosine-nitrated proteins intissue. Therefore, we used the IP followed by SDS-PAGE and Western blot analysis to determine whether aconitase, SDH, SOD are nitrated. MacMillan-Crow and Thompson were the first to describe a method for theimmunoprecipitation of nitrotyrosine-

containing proteins based on the use of a monoclonal anti-nitrotyrosine antibody followed by SDS-PAGE and Westernblot analysis. (245)

The proteins form small intestines were subjected to SDS PAGE electrophoresis and probed with specific antibodies (aconitase, SDH, and SOD) followed by immunoblot with anti-3NT antibody. Protein levels of these enzymes were not different between control and MTX. In the small intestines of MTX treated rats, aconitase, and SDH were shown to undergo more nitration as compared with control. However, to our surprise SOD, a protein that is proven to undergo nitration during inflammatory states did not show any difference in nitration between controls and MTX treatment.

Failure to demonstrate significant nitration of proteins after MTX treatment does not mean that nitration of these proteins does not occur as several factors affect PTN and hence the detection of nitrated proteins. Firstly, protein nitration is residue-, protein- and tissue-specific, with not all tyrosine residues of a protein being nitrated and not all proteins nitrated (107) (110), depending on cellular location of the protein and the peroxynitrite generating system, the concentration of peroxynitrite produced and interaction with other molecules. Secondly, the abundance of the protein and its tyrosine content have been proposed to influence protein nitration (110). The frequency of tyrosine occurrence in proteins is 3-4 mol%. Thridly, the location of the tyrosine residue (surface/packed within), neighbouring amino acids (particularly glutamic acid) (277), and presence of active site metals e.g. prostacyclin synthase (278) and heme proteins (279). Tyrosine nitration may also be favored in a hydrophobic environment due to the fact that peroxynitrous acid can readily pass through lipid membranes (280). Finally, some nitrated

proteins are transiently expressed while the amounts of other nitrated proteins could be decreased possibly through proteolytic degradation upon nitration. Therefore, it is difficult to capture and characterize all nitrated proteins analyzed at one time point.

As protein tyrosine nitration is typically a low-yield process and requires sensitive analytical methods for characterization. Nitrated proteins should be isolated or enriched before they are subjected to gel electrophoresis separation and protein digestion followed by final MS analysis to identify proteins and possibly Tyr-nitrated peptides. Several methods have been reported to detect Tyr nitration including, but not limited to: (1) two-dimensional gel electrophoresis followed by immunoblot analysis with specific anti-3-NT antibody (281), (2) immunoprecipitation with the specific antibody (282) or immunoaffinity chromatography using immobilized specific anti-3-NT antibody on Sepharose followed by capturing nitrated proteins (283), (3) solution isoelectric focusing, and (4) redox proteomic approach via conversion of nitro-Tyr to amino-Tyr followed by its labeling with biotin or dansyl chloride (268) to isolate nitrated proteins. Although indirect evidence is helpful in deciding whether a protein is nitrated, the gold standard is to sequence and identify the modified amino acid in the protein sequence (281-282).

Our study is the first one to investigate the effect of MTX on nitration of subcellular proteins in the intestinal mucosa and to identify the molecular weights of proteins that are nitrated. A limitation of this study is that we were unable to pinpoint the specific proteins that are affected. Until recently, the lack of specific enrichment and sensitive identification methods for nitrated residues has prevented the analysis of low-level protein modifications. Recently, tandem mass

spectrometry has been applied to the identification of nitrosative modification of tyrosine residues. These approaches have made it possible to identify several potentially important modification sites (284). In future, we plan to measure protein tyrosine nitration by immunoblot analysis using an anti-3-nitrotyrosine antibody in two dimensional PAGE separation and Western blot analysis, followed by mass spectrometric identification of immunopositive protein spots.

STUDY – II

Effect of MTX on the activities of enzymes that are established targets of peroxynitrite and are known to undergo nitration in inflammatory conditions

STUDY II: Effect of MTX on the activities of enzymes that are established targets of peroxynitrite and are known to undergo nitration in inflammatory conditions

6.2.1. ABSTRACT

During inflammatory states, many proteins have been shown to be nitrated by PON. In inflamed tissues nitration of mitochondria proteins have been shown to inactivate them and decrease their activity. Some of the PON target mitochondrial proteins are key enzymes in energy production; these include enzymes that are involved directly or indirectly in the citric acid cycle (e.g., aconitase, succinate dehydrogenase, glutamate dehydrogenase) and are involved in the electron transport chain (e.g., complexes I, II, complex IV (cytochrome oxidase) and Complex V(F1 ATPase) and energy distribution (e.g., creatine kinase). In addition to these mitochondrial proteins, four enzymes that protect cells against oxidative damage appear to be nitrated: MnSOD, catalase, glutathione *S*-transferase, and carbonic anhydrase III.

In the present study we assessed the effect of MTX on the activities of enzymes that are known to undergo nitration and inactivation. The above mentioned enzymes were assayed in the mucosal homogenates of control and MTX treated samples by spectrophotometric methods. The activities of ETC complexes: cytochrome c oxidase and F1-ATPase were decreased by 66% and 71% respectively in MTX rat intestines as compared to control. With respect to the activities of mitochondrial energy related enzymes, the activity of aconitase was decreased by 73%, succinate dehydrogenase by 85%, glutamate dehydrogenase by 61%, and creatine kinase by 71%, in MTX treated samples as compared with control. With regard to the antioxidant enzymes, superoxide

dismutase activity was increased and the activity of catalase was decreased by 68%, glutathione peroxidase by 60%, glutathione-s-transferase by 49% and carbonic anhydrase by 80% in MTX treated rat intestines as compared with control. This result indicates that MTX alters the activity of the enzymes that are known to undergo nitration and inactivation. MTX induced loss of ETC complex activity and depletion of antioxidant enzymes may contribute to mitochondrial injury, enterocyte apoptosis, and small intestinal injury.

6.2.2. INTRODUCTION

Protein nitration is a process that is found in normal tissues and is increased in disease states, mainly inflammatory states (106). A proteomic approach 2D-PAGE or LC-MS/MS toward targets of protein nitration in both in vivo and cell culture inflammatory disease models identified more than 40 nitrotyrosine-immunopositive proteins that became modified as a consequence of the inflammatory response (285). The biological functions and the subcellular locations of identified nitrated proteins are classified into multiple categories. Of the identified nitrated proteins in *in vivo* disease models, 25%, 20%, 28% are derived from mitochondria, extracellular, and cytoplasm or intracellular, respectively (285) .

Since, various active redox reactions occur in mitochondria, it is expected to be the center of nitration. These targets include proteins involved in oxidative stress, apoptosis, ATP production, and other metabolic functions. Mitochondria are essential for energy production and apoptosis, and both of these functions are affected by NO and related oxides. The major nitrated proteins identified are shown to be involved in energy metabolism (20%), (97, 200). Some of the PON target mitochondrial proteins are key enzymes in energy production; these include enzymes that

are involved directly or indirectly in the citric acid cycle (e.g., aconitase, SDH and glyceraldehyde phosphate dehydrogenase, glutamate dehydrogenase) and are involved in the electron transport chain (e.g., complexes I, II, complex IV (cytochrome oxidase) and Complex V (F1 ATPase) and energy distribution (e.g., creatine kinase). In addition to these mitochondrial proteins, four enzymes that protect cells against oxidative damage appear to be nitrated: MnSOD, catalase, glutathione S-transferase, and carbonic anhydrase III (286).

Protein tyrosine nitration alters the functions of proteins: the inhibition of function is a much more common consequence of protein tyrosine nitration (243). While gain of function as well as no effect on function are less common were reported for tyrosine nitrated proteins. Nitration of a tyrosine residue may also prevent further phosphorylation of that residue (287), thereby interfering with signaling pathways that are dependent on phosphorylation of tyrosine. Mitochondrial ETC complexes II, IV and F1 ATPase, glutamate dehydrogenase, aconitase, glyceraldehyde phosphate dehydrogenase, creatine kinase, SDH , MnSOD, catalase, and carbonic anhydrase III are examples of proteins that lose their catalytic activity upon nitration, and glutathione *S*-transferase and caspases are examples of enzymes that gain function upon tyrosine nitration (288-289).

Tyrosine nitration of mitochondrial proteins results in mitochondrial dysfunction through the impairment of catalysis and protein-protein interaction. The activities of complexes II, IV and V are significantly inhibited in ONOO--treated mitochondria (290). In the previous study (chapter 1) we have demonstrated increased PTN and differential nitration of proteins in the small intestines of MTX treated rats. PTN can result in a loss, an increase, or no effect on protein

function. In the present study we investigated the effects of MTX on the activities of the enzymes that are known targets of PON.

6.2.3. AIM and OBJECTIVES

The aim of the present study is to investigate the effect of MTX on the activities of enzymes that are targets of PON and are known to undergo nitration during inflammatory states

The objectives of the present study are to

- The effect of MTX on the activities of ETC complexes- cytochrome oxidase (complex IV) and complex V (F1 ATPase)
- Study the effect of MTX on the activities of key enzymes in energy production – Aconitase, Succinate dehydrogenase, Glutamate dehydrogenase , creatine kinase
- Study the effect of MTX on the activities of antioxidant enzymes, MnSOD, catalase, Glutathione peroxidase, glutathione *S*-transferase, and carbonic anhydrase III

6.2.4. SUMMARY OF EXPERIMENT

Adult male wistar rats (200 – 250g) were treated with MTX 7mg/kg body weight and the control rats were treated with vehicle alone intraperitoneally for three consecutive days. After 24 hours of the final dose of MTX the rats were sacrificed and the entire length of small intestine tissue and the mucosa scrapings was collected, stored for enzyme analysis. Mucosa samples were homogenized and 10% clear homogenate was used to assess alteration in the activities of enzymes that are known to be nitrated during inflammation including ETC complex enzymes, TCA cycle enzymes and antioxidant enzymes. All the enzyme assays was carried out by spectrophotometric method.

6.2.5. RESULT

MTX decreased the activities of ETC complexes

The activities of mitochondrial ETC complexes was decreased significantly, complex IV by 66% (0.209 ± 0.08 vs. 0.46 ± 0.11, P = 0.019) (Figure: 6.2.1), and F1ATPase by 71 % (0.106 ± 0.02 vs. 0.26 ± 0.07, P = 0.008) (Figure: 6.2.2) in the MTX treated rat intestine as compared with control.

MTX decreased the activities of TCA cycle enzymes and GDH

The activities of TCA cycle enzyme aconitase (Figure: 6.2.3) was reduced by 73 % (0.0026 ± 0.0002 vs. 0.006 ± 0.001, P = 0.010) and succinate dehydrogenase (Figure: 6.2.4) by 85% (0.0008 ± 0.0004 vs. 0.006 ± 0.001, P = 0.016) in MTX compared to control.

The activity of Glutamate dehydrogenase (Figure: 6.2.5), marker of mitochondrial injury was decreased by 61 % (0.036 ± 0.006 vs. 0.05 ± 0.006, P = 0.002) and Creatine kinase (Figure: 6.2.6) was decreased by 71 % (0.003 ± 0.0007 vs. 0.007 ± 0.001, P= 0.002) in the mitochondrial fractions of MTX treated rat intestines as compared with control.

MTX altered the antioxidant enzyme activity

To our surprise, the activity of Superoxide dismutase (Figure: 6.2.7), the guardian of the mitochondria was increased 40% (0.031 ± 0.007 vs. 0.02 ± 0.003, P = 0.015) in the small intestines of MTX treated rats as compared with control.

With respect to the activities of other antioxidant enzymes, catalase (Figure: 6.2.8) activity was decreased by 68 % (0.74 ± 0.13 vs. 2.98 ± 0.7, P = 0.029), Glutathione peroxidase (Figure: 6.2.9) activity was increased 60% (0.0047 ± 0.0002 vs. 0.0023 ± 0.0001, P = 0.025) and Glutathione s transferase (Figure: 6.2.10) by 49 % (1.78 ± 0.54 vs. 3.24 ± 0.8, P = 0.010) in the intestines of MTX treated rats as compare with control. Carbonic anhydrase (Figure: 6.2.11), an important antioxidant that prevent cell damage was 80 % (0.016 ± 0.006 vs. 0.04 ± 0.008, P = 0.010) decrease in activity in MTX treated rats compared to that of control.

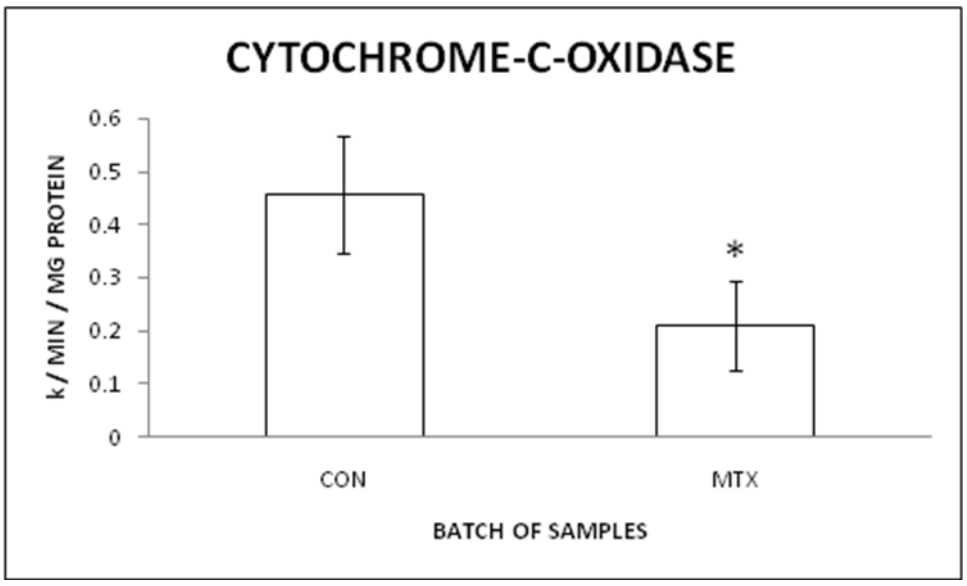


Figure: 6.2.1 - Effect of MTX on cytochrome-c-oxidase (CYT-C) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

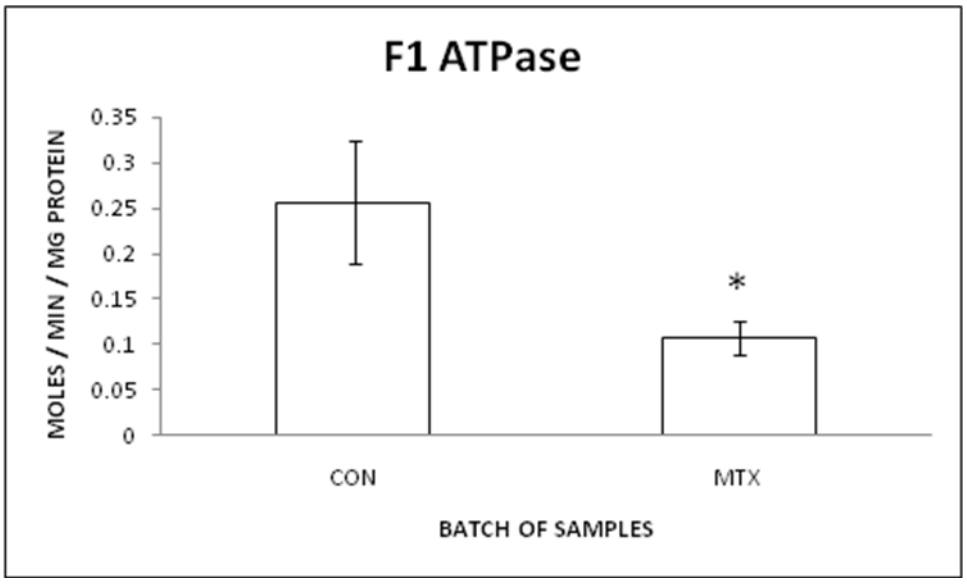


Figure: 6.2.2 - Effect of MTX on F1-ATPase (ATPase) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

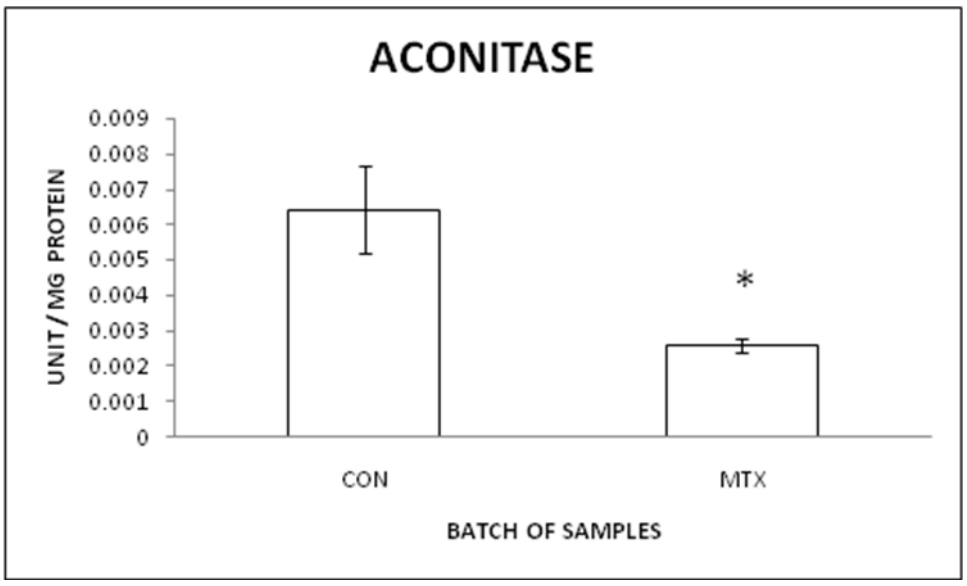


Figure: 6.2.3 - Effect of MTX on aconitase (ACO) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

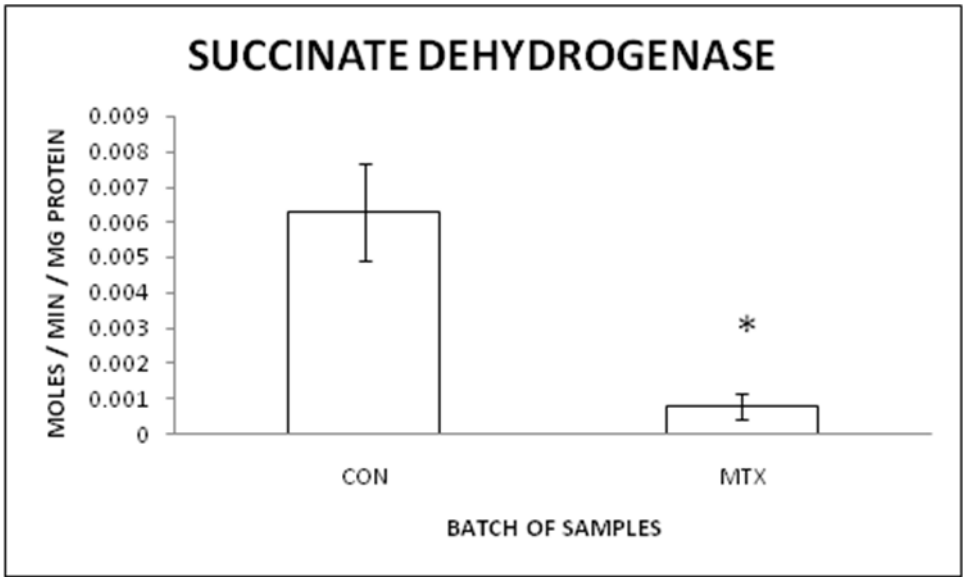


Figure: 6.2.4 - Effect of MTX on succinate dehydrogenase (SDH) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

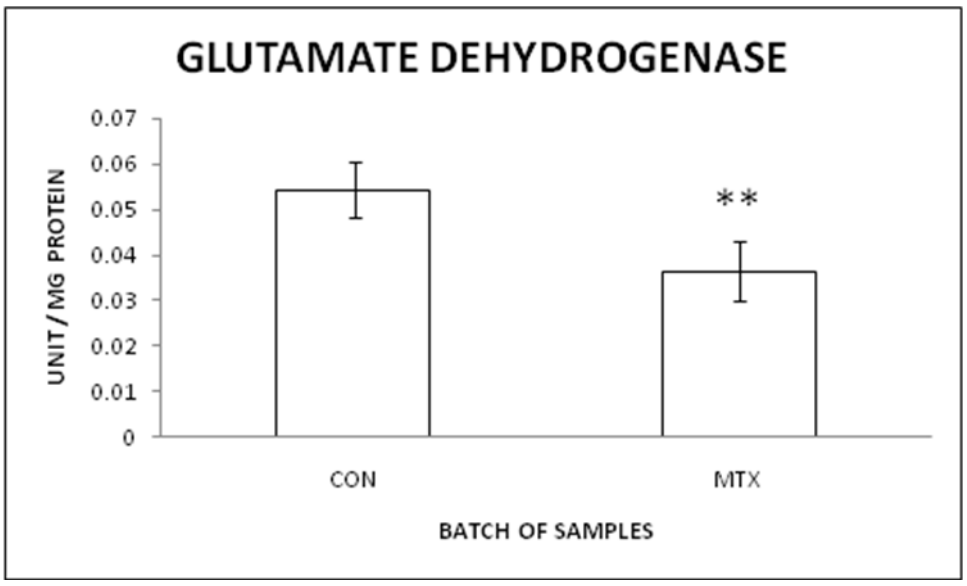


Figure: 6.2.5 - Effect of MTX on glutamate dehydrogenase (GDH) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control.

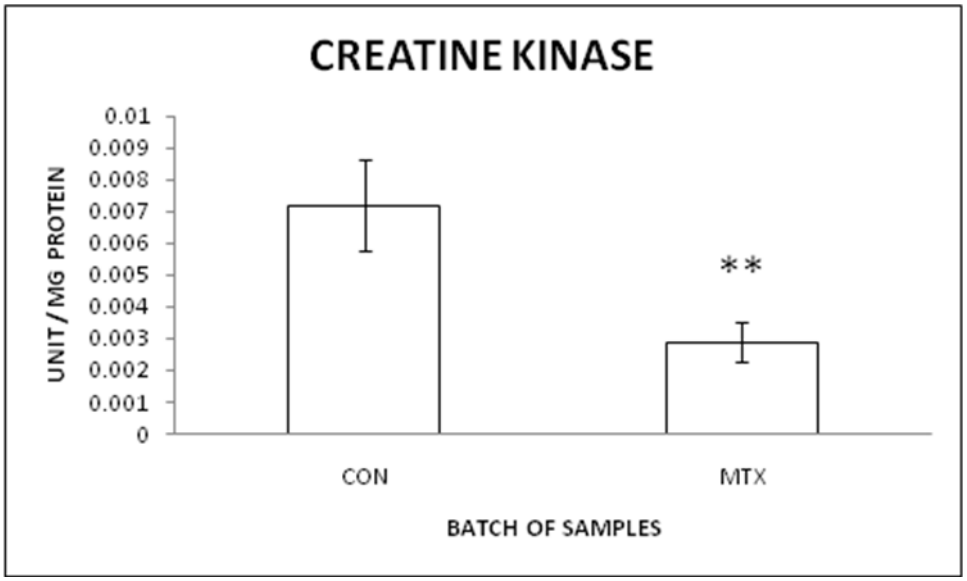


Figure: 6.2.6 - Effect of MTX on creatine kinase (CK) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control.

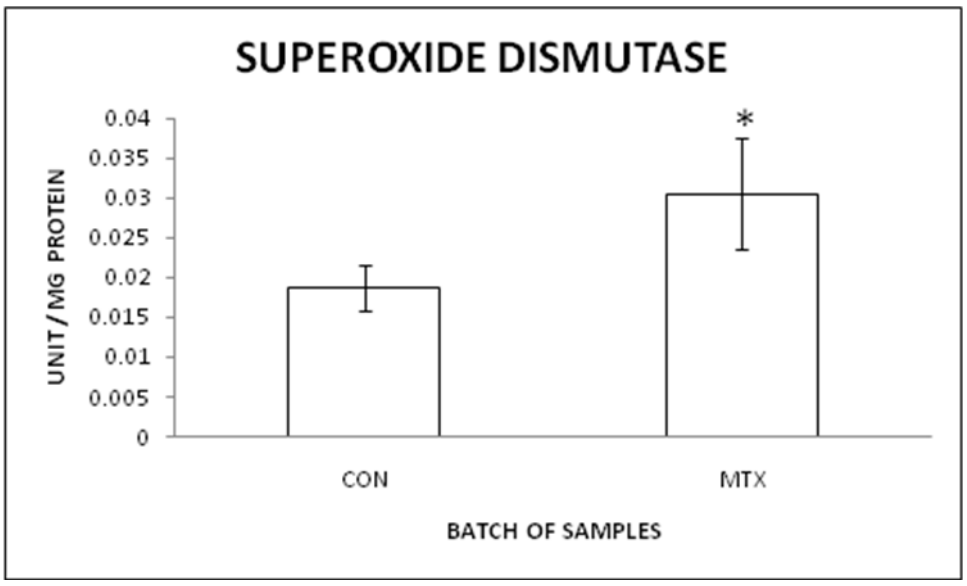


Figure: 6.2.7 - Effect of MTX on superoxide dismutase (SOD) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

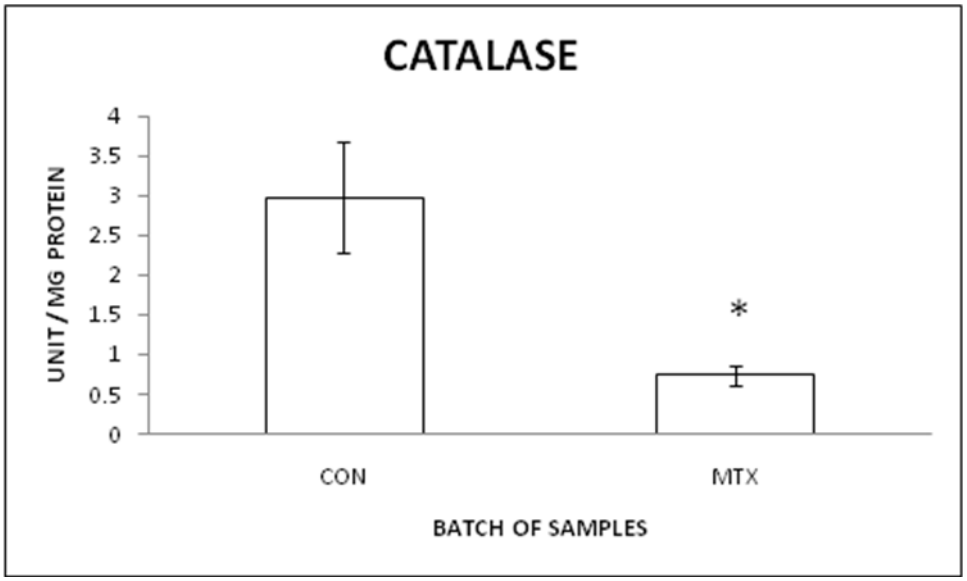


Figure: 6.2.8 - Effect of MTX on catalase (CAT) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

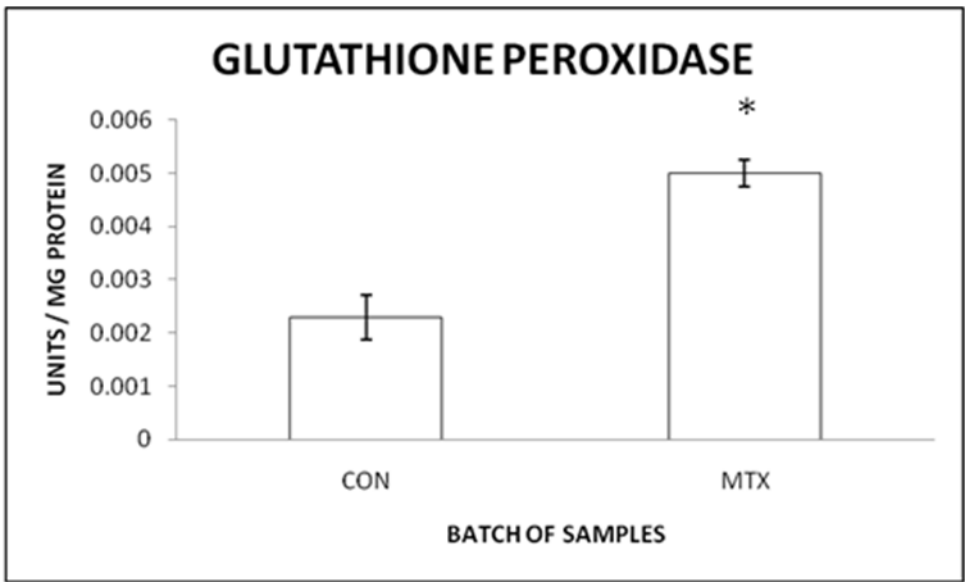


Figure: 6.2.9 - Effect of MTX on glutathione peroxidase (GPO) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

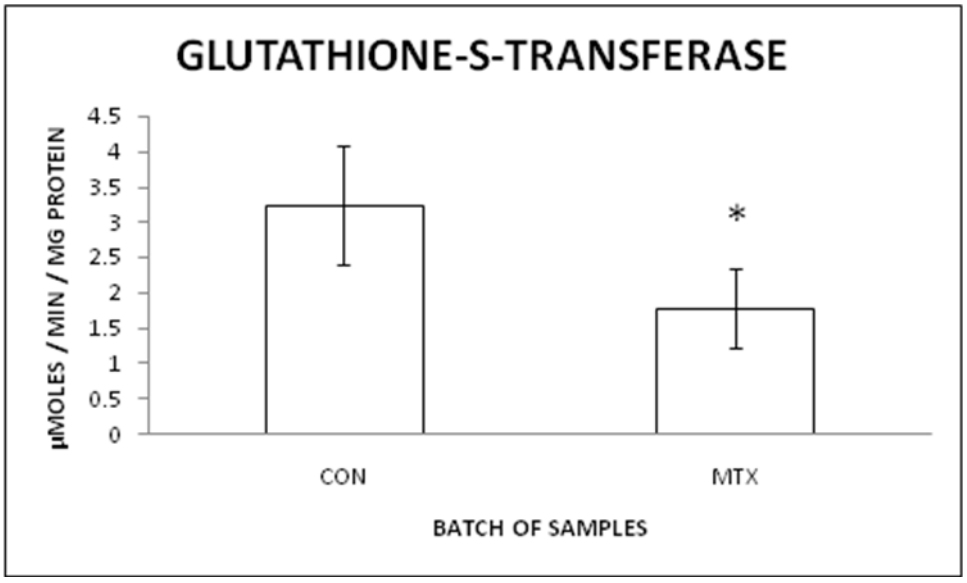


Figure: 6.2.10 - Effect of MTX on glutathione-s-transferase (GST) enzyme in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

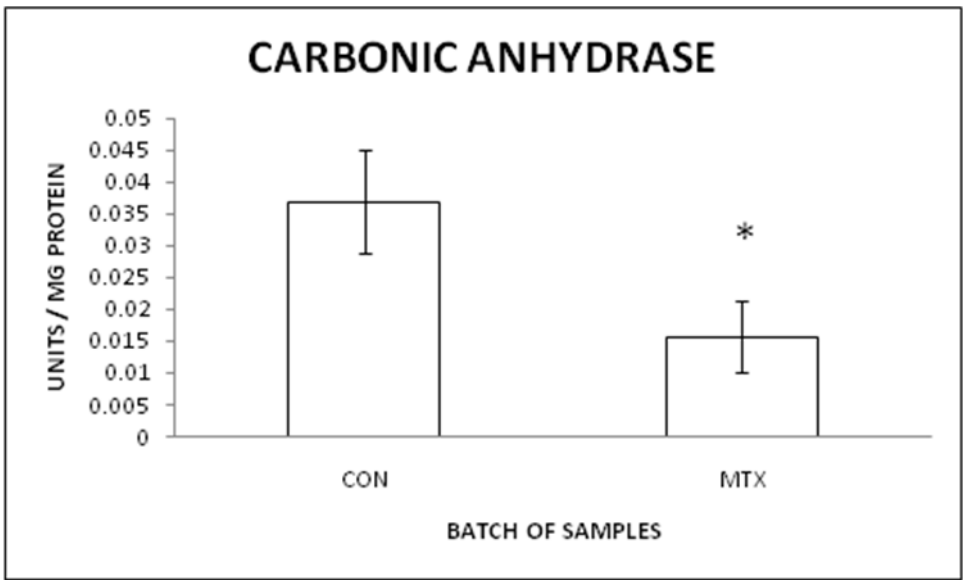


Figure: 6.2.11 - Effect of MTX on carbonic anhydrase (CA) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

6.2.6 DISCUSSION

During inflammatory conditions, among the proteins that undergo nitration over a third of the proteins that were identified are located in the mitochondria. Mitochondria are essential for energy production and apoptosis, and both of these functions are affected by NO and related oxides. Thus altered mitochondrial function may lead to cell death. While the reactions of NO with enzymes are usually eversible, the reactions of peroxynitrite are not. Through oxidation of critical cysteine residues, peroxynitrite irreversibly inactivates many components of the ETC, including complex II, complex III and complex V, leading to inhibition of oxidative phosphorylation and ultimately, cell death (291).

Some of the PON target mitochondrial proteins are key enzymes in energy production; these include enzymes that are involved directly or indirectly in the citric acid cycle (e.g., glutamate dehydrogenase, aconitase, and succinate dehydrogenase) and are involved in the electron transport chain (e.g., complex II, cytochrome oxidase (complex IV, and F1 ATPase) and energy distribution (e.g., creatine kinase). Therefore, we assayed the activities of these enzymes in mucosal homogenates.

In inflamed tissues, nitration of these and other mitochondria proteins have been shown to inactivate them and decrease their activity (108). In the present study we noted that the activities of Complexes IV and V were diminished significantly after MTX treatment 66% and 71%. Inhibition of complex IV may result in decreased mitochondrial respiration (115, 198). In addition, complex IV inhibition is predicted to increase ROS production, initially superoxide

anion, from electrons lost from the ETC. Studies have shown that excess NO inhibits cytochrome-c oxidase and superoxide may transiently leaks from the ETC, leading to the formation of peroxynitrite (292).

The activity of Complex V was decreased by 73% in MTX. ATP synthase in the mitochondrial electron transport chain catalyzes ADP phosphorylation to ATP. Mitochondrial ATP synthase is essential for providing cellular energy (i.e., ATP) for proper maintenance and survival of all living cells. ATP synthase/ATPase activity has been shown to be inhibited by tyrosine nitration (293). If ATP synthase (mitochondrial complex V) is inhibited, this leads to depletion of an essential energy source, thus contributing to necrotic tissue injury. The mitochondrial F1-ATP synthase α subunit is a component of the ATP generating complex V and is central to energy generation and able to respond to varying tissue energy demands. Immunoblot analysis with the anti-3-NT antibody following the immunoprecipitation of ATP synthase show that Complex V undergoes nitration and inactivation in different conditions such as in mice subjected to I-R injury, in MDMA exposed rats, in LPS-exposed *Ppara*(−/−)mice, and in mice exposed to a toxic dose of APAP or alcohol and mice with Alzheimers disease (294-296). It is noteworthy to mention that, in all of these models, the protein levels of ATP synthase seemed unchanged in response to any of those treatments, suggesting that the inhibition of its activity was mainly due to nitration mediated inactivation.

The nitration of ETC complexes appears to be agent specific. For instance, the 24 kDa subunit of complex I was shown to be 3-NT-immunopositive in diabetic mouse heart (297). During the process of aging, increased levels of nitration were observed in subunits from complex I,

complex III, complex IV and ATP synthase (298). Peroxynitrite reacts with mitochondrial membranes from bovine heart to significantly inhibit the activities of complexes I, II, and V (50-80%) but with less effect upon complex IV and no significant inhibition of complex III (290).

We next assessed the activities of enzymes that are reported to undergo nitration during inflammatory conditions, and are directly or indirectly related to energy production aconitase, SDH, GDH and Creatine Kinase in the small intestinal homogenates of control rats and MTX treated rats.

We found a significant decrease in aconitase activity in the MTX treated rat intestines as compared with controls. Aconitase appears to be the most sensitive of the studied mitochondrial enzymes to the effects of peroxynitrite (299). Aconitase, the TCA (tricarboxylic acid) cycle enzyme is a 4Fe24S cluster-containing protein that binds citrate and catalyzes its isomerization to isocitrate via the intermediate cis-aconitate. Aconitase also participates in cellular iron regulation and mitochondrial energy production by regulating the stability and translation of mRNA coding for proteins controlling as a function of iron availability (300). Decrease in aconitase activity is likely to affect the overall turnover efficiency of the citricacid cycle. Because intermediates produced in the citric acid cycle through anaplerosis do not accumulate in the mitochondria, and are diverted via cataplerosis, the citrate produced by citrate synthase would not be isomerized by aconitase and, consequently, would be exported. The accumulation of citrate in the cytosol would quite likely activate fatty acid synthesis. In addition, a decrease in aconitase activity would ultimately affect the products of the downstream reactions in the cycle.

PON is reported to in activate aconitase by two mechanisms. Peroxynitrite can bind to and directly nitrate the enzyme. ONOO−has been shown to provoke nitration of mitochondrial ACO2 at Tyr151 and Tyr472, both of which are located in close proximity to the active site of the enzyme. Nitration of these tyrosine residues has been postulated to cause conformational changes that destabilize the active site, thereby contributing to the ability of ONOO− to decrease ACO2 activity (301). In addition it can cause the disassembly of the cubane [4Fe-4S] cluster via its oxidative attack. Iron released from aconitase can further propagate intramitochondrial oxidative damage by metal-mediated formation of oxidizing and nitrating species. Evidence exista for in vivo PON induced nitration and loss of activity of aconitase in different pathological states that tyrosine nitration probably contributes to the decrease in renal mitochondrialACO2 activity evident in rats and mice with Type 1 DM (302), and in db/db mice (a model of Type 2 DM) despite more than 2-fold increase in renal cortical ACO2 protein levels (303). Interestingly, in the heart of diabetic rats, the overall activity of the TCA cycle is reduced due to ACO2 activation inthe reverse mode (isocitrate→citrate). This process relies on ACO2 phosphorylation at several sites, including Ser481, which may be influenced by nitration of the adjacent Tyr472 residue. (304)

With respect to the activity of SDH is one of the most important marker enzymes for mitochondria, as compared with control. Diminished SDH activity in diabetic rats affects the succinate-fumarate conversion, contributing to depressed oxidative metabolism in mitochondria. Satav and Katyare (305), the hepatic SDH activity was significantly decreased in STZ-induced diabetic rats.. It has been suggested that the diabetogenicity of STZ is due to the inhibition of the activities of citric acid cycle enzymes, such as SDH.

GDH activity was decreased by 61% in MTX treated rat intestines as compared with control. Glutamate dehydrogenase (GDH), located in the mitochondrial matrix, catalyzes the reversible deamination of glutamate to α-ketoglutarate, ammonium, and NADH. This α-ketoglurate can be channeled into the Kreb's cycle to stimulate ATP production. It is particularly useful in the truncated TCA cycle which cannot efficiently use glucose to generate energy (306). NADH can donate electrons to the electron transport system resulting in ATP production. In addition, GDH is also important in preventing accumulation of extracellular glutamate. GDH is known to contain seeral tyrosine residues that can undergo nitration. Bovine GDH contains 18 tyrosine residues, four of which (Tyr262, Tyr401, Tyr471 and Tyr493) are known to be susceptible to nitration (307). GDH is reported to undergo nitration and loss of activity in the livers of diabetic rats. Accumulation of tyrosine-nitrated GDH arises during conditions of sustained nitrosative stress, as evident in the liver during aging (308).

We assayed the CK and we found 70% decrease in the activity in the MTX treated rat intestines. Creatine kinase catalyzes phosphorylation of creatine by ATP at sites of ATP production, thus playing a key role in energy homeostasis with different isozymes localized at sites of energy production (mitochondria) and utilization (cytosol). Reduced activity of CK can reduce ADP supply to the mitochondrial ATP synthase and thereby compromise energy generation (309). Creatine kinase isoenzymes are very susceptible to free radical damage and are inactivated by superoxide radicals and peroxynitrite in vitro catalytic activity of creatine kinase is reduced by nitration. MS/MS analysis of nitrated peptides obtained from aging rat heart and skeletal muscle show that Tyr14 of creatine kinase undergoes nitration and enxyme becomes inactivated (310). Myofibrillar isoform of creatine kinase (a critical energetic controller of cardiomyocyte

contractility) is shown to be particularly sensitive target of peroxynitrite-induced nitration and inactivation in vivo (311).

MALDI-TOF MS and ESI-MS/MS analysis for the identification of nitrated proteins shown that the protein target for nitration depends on the agent or condition that induces nitrative stress. However, most of the the proteins that are targeted are those involved in energy metabolism. Western blot showed an age-related increase in the nitration of beta-enolase, alpha-fructose aldolase, and creatine kinase, succinate dehydrogenase, rab GDP dissociation inhibitor beta (GdI-2), triosephosphate isomerase, troponin I, alpha-crystallin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which perform important functions in muscle energy metabolism (312). Nitrated proteins in renal tissue from sepsis-induced AKI rats were glutamate dehydrogenase, methylmalonate-semialdehyde dehydrogenase, and aldehyde dehydrogenase, mitochondrial proteins involved in energy metabolism or antioxidant defense (313). In cardiac tissues of aged Fisher 344/Brown Norway F1 rats total of 48 nitrated proteins were putatively identified. Among the identified proteins, there are important enzymes responsible for energy production and metabolism as well as proteins involved in the structural integrity of the cells. The identified proteins were alpha-enolase, alpha-aldolase, desmin, aconitate hydratase, methylmalonate semialdehyde dehydrogenase, 3-ketoacyl-CoA thiolase, acetyl-CoA acetyltransferase, GAPDH, malate dehydrogenase, creatine kinase, electron-transfer flavoprotein, manganese-superoxide dismutase, F1-ATPase, and the voltage-dependent anion channel (314).

Oxidative stress and inflammation are tightly correlated. Pathways that generate mediators of inflammation (e.g., adhesion molecules, and interleukins) are induced by oxidative stress (53, 315). Antioxidant enzymes are considered to be the first line of cellular defense against oxidative damage. Intestinal inflammation is accompanied by excessive production of reactive oxygen and nitrogen metabolites. In order to counteract their harmful effects, the intestinal mucosa contains an extensive system of antioxidants A non-harmful concentration of ROS/RNS is sustained by the anti-oxidative defence mechanisms, that include enzymes such as CAT, SOD or GPx and non-enzymatic endo- and exogenous scavengers like glutathione (GSH), transient ions (e.g. Fe^{2+}, Cu^{2+}) or flavonoids.

SOD is normally present at low micromolar concentrations in cells. This makes SOD-catalyzed dismutation of superoxide very feasible. SOD can act as an antioxidant by different mechanisms. By decreasing the intracellular steady state levels of superoxide, SOD could successfully inhibit the reaction between $O_2^{\bullet-}$ and $^{\bullet}NO$ and the formation of a potent oxidant, $ONOO^-$. In addition, SOD could prevent the release of iron ions from the iron–sulfur cluster enzymes, as superoxide is known to rapidly react with the iron–sulfur centers and release iron. Thus, SOD can also indirectly prevent the formation of hydroxyl radical from the Fenton reaction (316). Three mammalian SOD isoforms, copper/zinc (SOD1), mitochondrial (SOD2) and extracellular (SOD3), catalyse the reaction of $O_2^{\bullet-}$ reduction to H_2O_2. SOD1 is a cyanide-sensitive homodimer localized mainly in the cytoplasm and to some extent in the nucleus, but absent in the mitochondria of epithelial cells and phagocytes. The mitochondria are protected from $O_2^{\bullet-}$ by SOD2, which is vital for cell survival as mice lacking SOD2 gene die within several days after

birth. SOD3 dominates in plasma and interstitium and has a high affinity to glycosaminoglycans like heparin (317).

Manganese superoxide dismutase (MnSOD) provides the first line of defense against superoxide generated in mitochondria SOD competes with nitric oxide for reaction with superoxide and prevents generation of peroxynitrite, a potent oxidant that can modify proteins to form 3-nitrotyrosine. Thus, sufficient amounts of catalytically competent MnSOD are required to prevent mitochondrial damage. Manganese superoxide dismutase (Mn-SOD), a critical mitochondrial antioxidant enzyme, becomes inactivated and nitrated in vitro and potentially in vivo by peroxynitrite (318). Tyrosine 34 of human MnSOD was exclusively nitrated to 3-nitrotyrosine and almost completely inactivated by the reaction with peroxynitrite. The nitrated MnSOD has been found in many diseases caused by ischemia/reperfusion, inflammation, and others and may have a pivotal role in the pathology of the diseases. Most of the post-translational modifications have given rise to a reduced activity of SOD. Since phosphorylation and nitration of SOD have been shown to have a possible reversible process, these modifications may be related to a redox signaling process in cells (319). In our study, we found significant increase in MnSOD activity in the intestines of MTX treated rats as compared to control. This observation is rather surprising as several studies have shown this posttranslation nitration of SOD results in its inactivation and decreased activity in various conditions including ischemia/reperfusion, inflammation, and others and it is suggested that it may have a pivotal role in the pathology of the diseases (320).

PTN and inactivation of SOD have been reported in several models of tissue toxicity. The inactivation of MnSOD, by tyrosine nitration represents a pathophysiological cellular mechanism contributing to self-perpetuation and amplification of CsA-related vascular toxicity (321). LPS administration also resulted in a rapid and significant loss of more than 80% of pulmonary EC-SOD in a time- and dose-dependent manner, but other types of SODs, cytoplasmic CuZn-SOD and mitochondrial Mn-SOD, were not affected (322). It has been reported that MnSOD is a target of tyrosine nitration after traumatic brain injury (TBI) in mice and that is associated with a decrease in its enzymatic activity. The authors have suggested that nitration and inactivation of MnSOD could lead to self-amplification of oxidative stress in the brain progressively enhancing peroxynitrite production and secondary damage (323). Furthermore, oxidation and nitration of MnSOD were identified in the asthmatic airway, correlating with physiological parameters of asthma severity. These findings link oxidative and nitrative stress to loss of SOD activity and downstream events that typify asthma, including apoptosis and shedding of the airway epithelium and hyper-responsiveness (324).

The activity of SOD is known to serve protective function for the elimination of reactive free radicals and thus it represents an important antioxidant defense in nearly all cells exposed to oxygen (316) and therefore we propose that increase in the activity of SOD after MTX treatment may be a defense mechanism. SOD is an interesting enzyme since at low levels, the SOD enzymes are anti-inflammatory, whereas at high doses they exhibit proinflammatory effects (325). Under normal conditions, the superoxide anions produced from the ETC will be dismutated to hydrogen peroxide, thus making superoxide unavailable for peoxynitrite formation. The H_2O_2 formed by the SOD will be detoxified by catalase and glutathione

peroxidases. Thus low levels of SOD are anti-inflammatory. The proinflammatory effects of the SOD enzyme are not well understood, but it is speculated to be due to its production of high amounts of the reactive oxidizing agent hydrogen peroxide which in the presence of iron results in the production of the very toxic hydroxyl radical via the Fenton reaction (326). In contrast to $O_2^{\bullet-}$, H_2O_2 is more stable and could freely diffuse to other compartments in cells. Under conditions by which an excess of superoxide anion is produced, and where iron is available we may suddenly see the SOD functioning more as a generator of hydroxyl radicals rather than as a primary scavenger of superoxide radicals. Thus, overexpression of SOD may exacerbate the tissue damage by producing excessive H_2O_2. Thus increased activity of SOD in the small intestines of MTX treated rats observed in the present study may be a defense mechanism to minimize PON formation and hence tissue injury or it may exacerbate the tissue damage by producing excessive H_2O_2 and depleting the physiological levels of NO (320, 326).

H_2O_2 (hydrogen peroxide) is a strong membrane passing oxidant that is rapidly eliminated from the cell to prevent induction of oxidative damage to lipids, proteins and DNA. H_2O_2 is detoxified to water by catalases and peroxidases (327). Catalase detoxifies hydrogen peroxide and is one of the well-known antioxidant enzymes. The conjugation of catalase / glutathione peroxidase to SOD appears to ensure that as soon as a superoxide dismutation reaction occurs, the resultant H_2O_2 is removed by the immediate proximity of the catalase / peroxidase molecule (328). CAT is widely expressed in the cytoplasm and peroxisomes of GI epithelium and lamina propria and activated when concentrations of H_2O_2 increase, e.g. during inflammatory process (98). Therefore we assayed the activitiy of catalase and shown to be decreased by 68% in MTX treated rat intestine.

Catalase deficiency may lead to accumulation of reactive oxygen metabolites and this may cause the initiation of inflammation and tissue injury. Catalase is a priority enzyme of the antioxidant system in defense for oxidative stress occurring in many pathological conditions including cancer, diabetes, cataract, atherosclerosis, ischemic-reperfusion damage, arthritis, neuro-degenerative disease, nutritional deficiency, and aging (329-330).

GPx has higher affinity to H_2O_2 than CAT and also reduces lipid hydroperoxide levels, preventing peroxynitrite-mediated oxidation (331). GPx enzymatic activity requires glutathione as a proton donor. Currently, five isoforms of GPx, have been shown to be expressed in GIT. GPx1 and GPx2 play an important role in the intracellular antioxidant defence, but in different layers of the gut; GPx1 is highly expressed in the lymphatic tissue and the lamina propria, submucosa, muscularis and serosa, but not the luminal epithelium, which is the area of the action of GPx2. GPx3 most likely contributes to the extracellular antioxidant defense of the intestinal mucosa, as it is secreted by intestinal epithelial cells (332). Recently, GPx4 has been detected in colonic and ileal tissues (333). This isoform is responsible for a repair of oxidatively damaged DNA by reducing thymine hydroperoxide and for scavenging phospholipid hydroperoxides and repressing lipid peroxidation. In the present study we were able to detect GPO by 2 fold in MTX. Studies have shown that both catalase and GPx can be inactivated by $NO^{\bullet}$, peroxynitrite or diminished GSH levels (334).

It is suggested that catalase/GPO itself is without significant effect, since the H2O2 accumulation is low in the absence of SOD. Ferric ion has a similar catalytic effect but at concentrations 2 -3 orders of magnitude higher. Only at these higher concentrations will the iron compete with SOD

for available superoxide as a reductant. In our study an increase in SOD activity was accompanied by a drastic decrease in the activity of catalase and GPO in the small intestines of rats after MTX treatment. This suggests that the overproduction of H_2O_2 by increased SOD activity, and the underutilization of H_2O_2 by decreased catalase activity may contribute to MTX induced small intestinal injury.

GST proteins, another important class of mucosal antioxidant defense are abundant in the gastrointestinal tract, GST distribution varies considerably along the digestive tract (mainly in colon and to lesser extent in the small intestines) and their activity and expression is influenced by diet, drug exposure, and clinical conditions (335). In the present study GST activity in the MTX treated rat intestines was significantly decreased as compared with control .Studies have shown that the microsomal glutathione S-transferase 1 is activated rather than inactivated by peroxynitrite and suggested that this could be attributed to nitration of tyrosine residues rather than to other effects of peroxynitrite. The activation of microsomal glutathione S-transferase 1 by peroxynitrite is mediated by nitration of tyrosine residue 92 and represents one of the few examples in which a gain in function has been associated with nitration of a specific tyrosine residue (336). Our findings are in agree with two other studies that have been reported, However, two other study show nitration and inactivation. Treatment of isolated GSTs or rat liver homogenates with either peroxynitrite, resulted in loss of GST activity with a concomitant increase in the formation of protein-associated 3-nitrotyrosine (NO) Tyr (337).

Studies have shown that carbonic anhydrase (CA) (mainly CA III) could have a direct role in cellular response to oxidative damage. Therefore, we assayed the activity of CA in the small

intestinal tissues. CA was decreased. CA III is abundant in tissues such as skeletal muscle, which may make it physiologically a significant pool of reactive sulfhydryls that function as oxyradical scavengers. It has been shown that the concentration of CA III in these cells could reach the same order of magnitude as that of glutathione. Chatterjee et al. have described a protein (senescence marker protein-1) that is present in high amounts in adult rat liver and decreases during aging. This protein was later identified to be CA III (338). Carbonic anhydrase III (CAIII) functions as an anti-oxidant agent in skeletal muscle. Thus CAIII responds to oxidative stress with a distinctive sulfhydryl oxidation patterns reflecting duration and severity that may prove sensitive indices of extent and type of damage in muscle injury (339). S-thiolated forms of CA III were also detected in rat skeletal muscle and heart showing the utility of this method for determining the effect of oxidative stress on specific S-thiolatable protein in several tissues *in vivo* (340).

In this study we show decreased activity of enzymes that are susceptible to nitration by PON in the small intestines of MTX treated rats. However, we have not demonstrated that the loss of activity is due to protein tyrosine nitration. Protein tyrosine nitration is not the only mechanism by which enzymes get inactivated. There are other mechanisms such as cysteine nitrosylation, lipid peroxidation, loss of metal ion from active site that are known to inactivate these enzymes. In future, we plan to investigate whether there is any causal relationship between protein tyrosine nitration and loss of activity of the enzymes studied.

STUDY – III

Role of inflammatory pathway in methotrexate induced small intestinal injury

STUDY III: Role of NFκB-iNOS-COX-TNFα inflammatory pathway in methotrexate induced small intestinal injury

6.3.1. ABSTRACT

As a major transcription factor and a first responder to harmful cellular stimuli, NF-κB plays a central role in inflammation through its ability to induce transcription of proinflammatory genes. Reactive oxygen and nitrogen intermediates are thought to be important in activation of the redox-sensitive transcription factor, NF-κB. In chap 1we have demonstrated increase protein tyrosine nitration in the small intestines of MTX treated rats. Studies have shown that tyrosine nitration triggers dissociation of IκB from NuclearFactor-κB (NF-κB), which results in activation of this pathway that plays an important role in cancer and inflammation. NF-κB controls the activity of numerous genes crucial for immunity, inflammation, and stress responses, including TNFα, NOSII, COX-2, and $cPLA_2$. Therefore, we investigated the role of NFκB inflammatory pathway in MTX induced mucositis. Adult wistar rats were treated with MTX 7mg/kg body weight and control rats with vehicle for 3 consecutive days. After 24 hours of the final dose of MTX rats were sacrificed and the small intestine was removed. A piece of small intestinal segments was stored for immunohistochemistry, immunofluorescence studies and the mucosa was collected and stored for the western blot, RT-PCR analysis.

MTX treatment resulted in NFκB activation and nuclear translocation as evidenced by immunofluorescence, immunohistochemitry and immunoblot analysis of intestinal segments. Western blot analysis revealed 50% increase NFkB expression in nucleus and 55% decrease in cytosol fraction in MTX when compared to control. Gene transcription of NFκB assayed by RT

PCR revealed significant increase more than 50%. The protein and mRNA expressions of NFκB target genes TNF-α, iNOS, COX-2, PLA_2, HO-1, HSP70, MMPs 2 and 9 were determined by IHC, WB and RTPCR. There was increased immunostaining of COX2 and PLA_2 in MTX treated rat intestines. We found 75% increase in TNFα and 70% increase COX2 protein expression in MTX treated rats as compared to control. RT-PCR revealed significant increase in gene expression: TNFα by 60%, iNOS by 55%, COX2 by 80%, HO-1 by 90%, HSP70 by 75%, MMP2 by 78% and MMP9 by 40% in MTX as compared to control. Protein expression of MMP2 was increased by 65% and MMP9 by 50% in MTX treated samples as compared to control. In conclusion, this study demonstrates that activation of NFκB signaling inflammatory pathway plays a role in MTX treated rats.

6.3.2. INTRODUCTION

Inflammation is a protective response to cellular and tissue damage/injury. The purpose of this process is to destroy and remove the injurious agents and injured tissues, thereby promoting tissue repair. When this beneficial response occurs in an uncontrolled manner, the result is excessive cellular/tissue damage that results in chronic inflammation and destruction of normal tissue. Many of the known inflammatory target proteins, such as matrix metalloproteinase-9 (MMP-9), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), (COX-2), and ($cPLA_2$), are associated with inflammatory signaling pathways induced by various stimuli, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), adenosine-5-triphosphate (ATP), , lipoteichoic acid (LTA), or lipopolysaccharide (LPS) (132).

Once $ONOO^-$ forms, it can act via several distinct mechanisms; first it has direct toxic effects leading to lipid peroxidation, protein oxidation and DNA damage. The second mechanism

involves the induction of several transcription factors including nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) leading to cytokine induced chronic inflammation. This cascade, once activated, causes the release of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) andinterleukin-1β (IL-1β), and a variety of chemokines which induces widespread inflammation (114, 341).

As a major transcription factor and a first responder to harmful cellular stimuli, NF-κB plays a central role in inflammation through its ability to induce transcription of proinflammatory genes (127). NF-κB controls the activity of numerous genes crucial for immunity, inflammation, and stress responses, including TNF-α, NOSII, COX-2, and cPLA. Reactive oxygen and nitrogen intermediates, and cytokines such as TNF are thought to be important in activation of the redox-sensitive transcription factor, NF-κB (130). Studies have shown that tyrosine nitration triggers dissociation of IκB from NuclearFactor-κB (NF-κB), which results in activation of this pathway that plays an important role in cancer and inflammation (111, 131). Tyr-66 and Tyr-152 of p65 subunit of NFκB have been shown to undergo nitration (342-343).

NFκB has been studied extensively since its first description by (126) for its role in immunity and stress responses. NFκB is normally stabilized in the cytosol when bound to its inhibitor protein, IκB to form p50/p65 IκB complex. Activation of the IκB kinase complex by numerous stimuli leads to the phosphorylation of IκB, causing its dissociation from the NF-κB heterodimer and subsequent degradation by the proteasome (344) leaving NFκB available for translocation to the nucleus and activation of target genes. Some target genes for NFκB include pro-inflammatory cytokines such as , cyclooxygenase-2 (COX-2),tumor necrosis factor (TNF)-α,

iNOS, MMP 9, HO-1,interleukin (IL)-1β and IL-6, which in turn lead to tissue injury and apoptosis (345).

Persistent activation of NF-κB signaling pathways is often associated with chronic inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and asthma (133). NF-κB activity and nuclear localization have been consistently detected in biopsies from such patients, and are accompanied by enhanced recruitment of inflammatory cells and production of proinflammatory mediators such as IL-1, IL-6, and TNF (133, 346). Genetic manipulations that lead to increased NF-κB activity often trigger inflammation-related pathologies (347-348). Other studies confirmed that NF-κB inhibition has antiinflammatory effects in vivo (349).

It has been postulated that mucositis occurs in five overlapping phases that occurs sequentially: initiation, up-regulation and message generation, signaling and amplification, ulceration and inflammation and healing phase (69). During the second phase, significant up-regulation of inflammatory mediators is observed and nuclear factor kappa-B (NF-κB) is thought to be pivotal in this process. Upon activation by an extensive range of agents such as bacteria and bacterial cell wall products, viruses, cytokines, free radicals such as ROS and RNS,, ionizing radiation, and even the use of anti-neoplastic agents (cisplatin, doxorubicin, taxol, paclitaxel, and etoposide), NF*κ*B acts to induce gene expression of many cytokines involved predominantly in mucosal inflammation, and angiogenesis, chemokines, immunoreceptors, cell adhesion molecules, proapoptotic and antiapoptotic as well as stress response genes (60, 67, 350-351). Nuclear factor kappa B (NFκB), cyclooxygenase-2 (COX-2) as well as pro-inflammatory

cytokines (in particular interleukin (IL)-1β (IL-6) and tumour necrosis factor (TNF)) have been suggested to play a key role in this 5 phase mucositis model.

In the previous studies we have demonstrated ROS and RNS and protein tyrosine nitration play important roles in MTX induced enteritis (25). As NF-κB is a redox-sensitive transcription factor that can be modulated by tyrosine nitration and as nuclear factor kappa-B (NF-κB) is thought to be pivotal role during the second phase of mucositis, the present study is aimed at investigating in detail the role of NFκB inflammatory pathway in MTX induced mucositis.

6.3.3. AIM and OBJECTIVES

The present study was designed to examine the role of NFκB-iNOS-COX-2 TNFα inflammatory signaling pathway in MTX enteritis.

The objectives of this study is to investigate the effect of MTX on

- NFκB activation - NFκBp65 protein expression and mRNA expression by immunofluorescence, and western blot and immunohistochemical methods and quantitive real time PCR respectively
- COX-2 and TNF gene expression and protein expression by qRT PCR and western blot and IHC respectively as well as PLA_2 by IHC.
- MMP2 and MMP9 gene expression and protein expression by qRT PCR and western blot rspectively
- HSP 70 and heme oxygenase-1 mRNA expression by qRT PCR

6.3.4. SUMMARY OF EXPERIMENT

Adult male wistar rats (200 – 250g) were treated with MTX 7mg/kg body weight and the control rats were treated with vehicle alone intraperitoneally for three consecutive days. Twenty four hours after the final dose of MTX/saline the rats were sacrificed and the entire length of small intestine tissue was removed, washed with saline and a piece of segments (duodenum, jejunum, ileum) was stored for immunohistochemistry and immunofluorescence studies. The mucosa scrapings was collected and used for the isolation of cell fractions by ultracentrifugation and also for the isolation of RNA for RT-PCR studies. The gene expression of TNF α, NFκB, iNOS, COX-2, HO-1, HSP70, MMP2, MMP9 was done by RT-PCR. The cell fractions were used for the western blot of TNF α, NFκB, COX-2, MMP2 and MMP9. Immunofluorescence of NFκB and immunolocalisation of NFκB, COX-2, PLA_2 was done by immunohistochemistry.

6.3.5. RESULT

MTX resulted in activation and nuclear translocation of NFκB as assessed by Immunofluorescence, Immunohistochemistry, Western blot and RT PCR

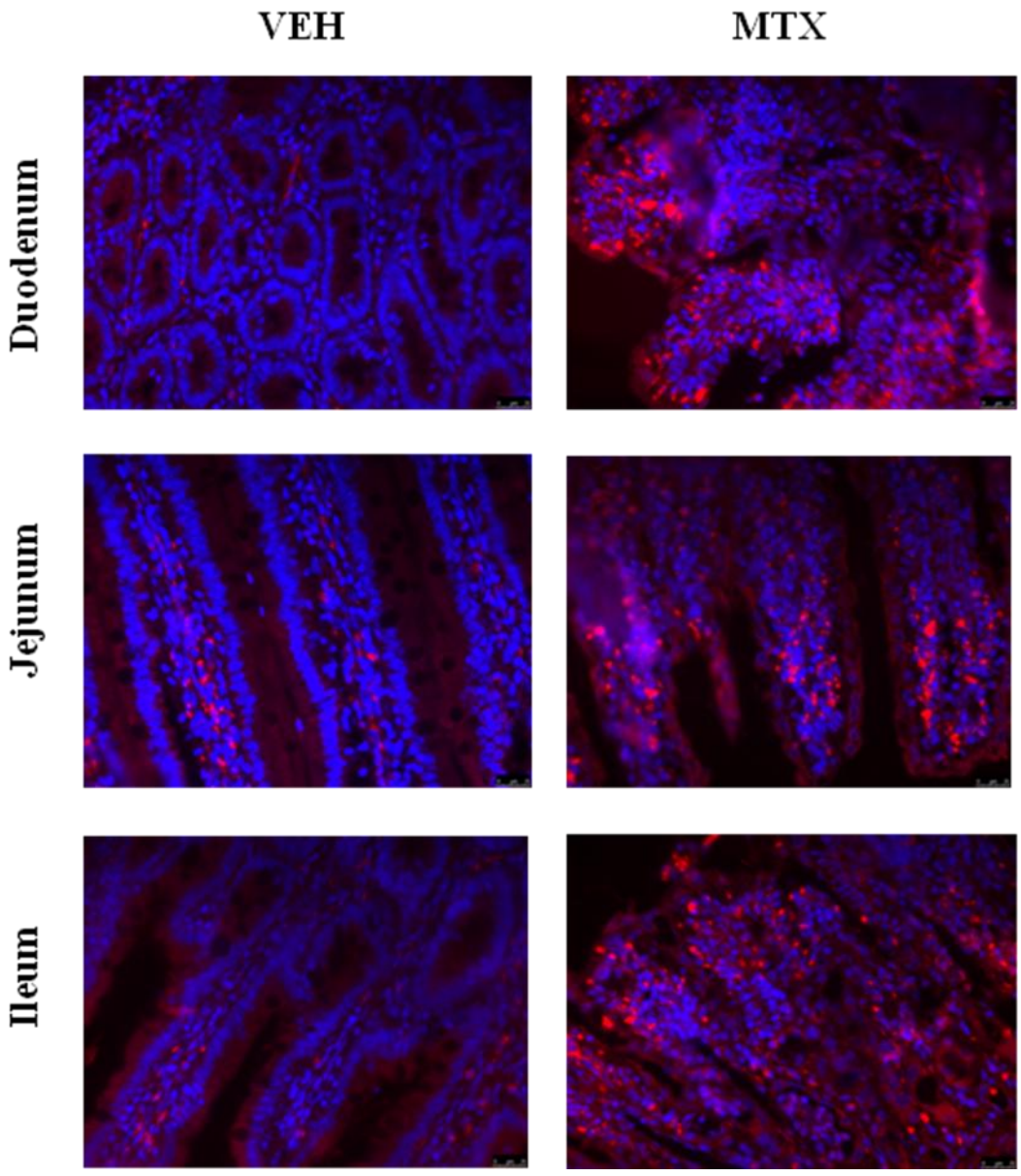
The nuclear transcription factor NF-κB is a key regulator of the inducible expression of many genes involved in immune and inflammatory response in the gut. The expression and location of NF-κB p65 was examined by IF, immunohistochemistry and western blot in small intestinal tissue. Immunofluorescence showed increased NFκB expression in the nucleus in all small intestine segments duodenum, jejunum and ileum of MTX treated rats when compared to control (Figure: 6.3.1). The intensity of p-65 immunohistochemical staining was used to assess NF-κB activation indirectly in intestinal tissue from rats treated with MTX. NF-*κ*B p65 protein was detected throughout the cytoplasm of crypt epithelial cells and surface epithelium, suggesting that NF-κB is constitutively active at low levels in the small intestines, whereas a high expression of nuclear factor appeared in intestinal mucosa of MTX treated rat, as evidenced by increased intensity of brown staining. Immunolocalization of NFκB (Figure: 6.3.2) revealed increased expression in duodenum, jejunum and ileum of MTX as compared to control. There were many brown p65-reactive cells in the crypts and villi of-MTX-treated intestine. NF-κB p65 was mainly expressed in the nucleus, to lesser extent in the cytoplasm of epithelia and macrophages.

Nuclear translocation of NFκBp65 is often recognized as cell reaction to ROS and cytokine stimulation and correlate with NF-κB-mediated transcriptional activation. So, we measured the level of NF-κB p65 protein in the nucleus (Figure: 6.3.3) and cytosolic (Figure: 6.3.4) fractions of intestinal homogenates using western blot. In the control rats the amount of NFκB in the

cytoslic fraction was higher as compared with nuclear fraction, suggesting that most of the NFκB is in the resting state. MTX treatment resulted in nearly a two fold increase in p65 levels in the nucleus (1.93 ± 0.96 vs. 0.90 ± 0.17) as compared with cytosol (0.75 ± 0.21 vs. 1.67 ± 0.26) suggesting the activation of NFκB pathway after MTX treatment.

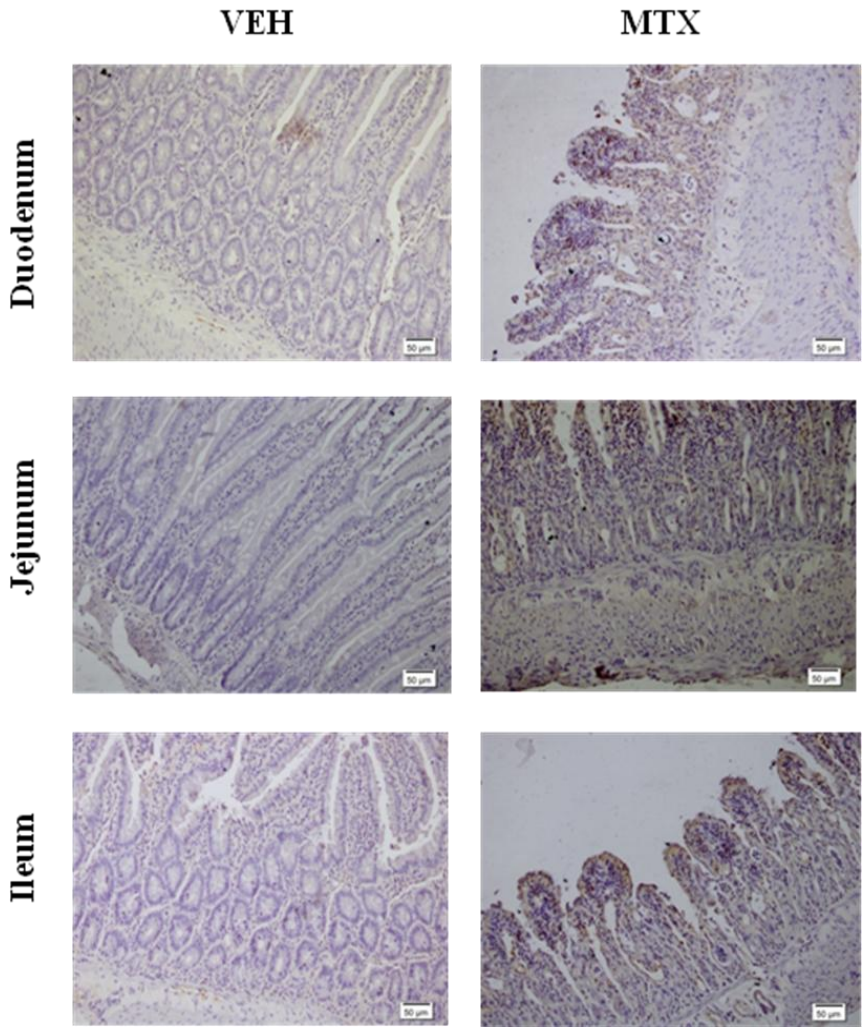
To demonstrate the effect of MTX on NF-κB gene expression, we utilized real-time quantitative PCR. The results indicate that mRNA expressions of NF-κB p65 was significantly increased 1.65 fold (0.009 ± 0.003 vs. 0.004 ± 0.001, P = 0.016) in MTX-treated rats as compared with controls. (Figure: 6.3.5).

Figure: 6.3.1 **NFκB translocation in nucleus by IF**



Representative image of nuclear factor kappa B (NFκB) expression that reveal nuclear translocation in the small intestine segments of rats 24 hours after the treatment with vehicle and MTX by immunofluorescence analysis (40X). MTX treated rats shown increased immunofluorescnece of NFκB expression (red) as well as nuclear (blue) translocation when compared to basal immunostaining in control rat small intestine tissue treated with vehicle.

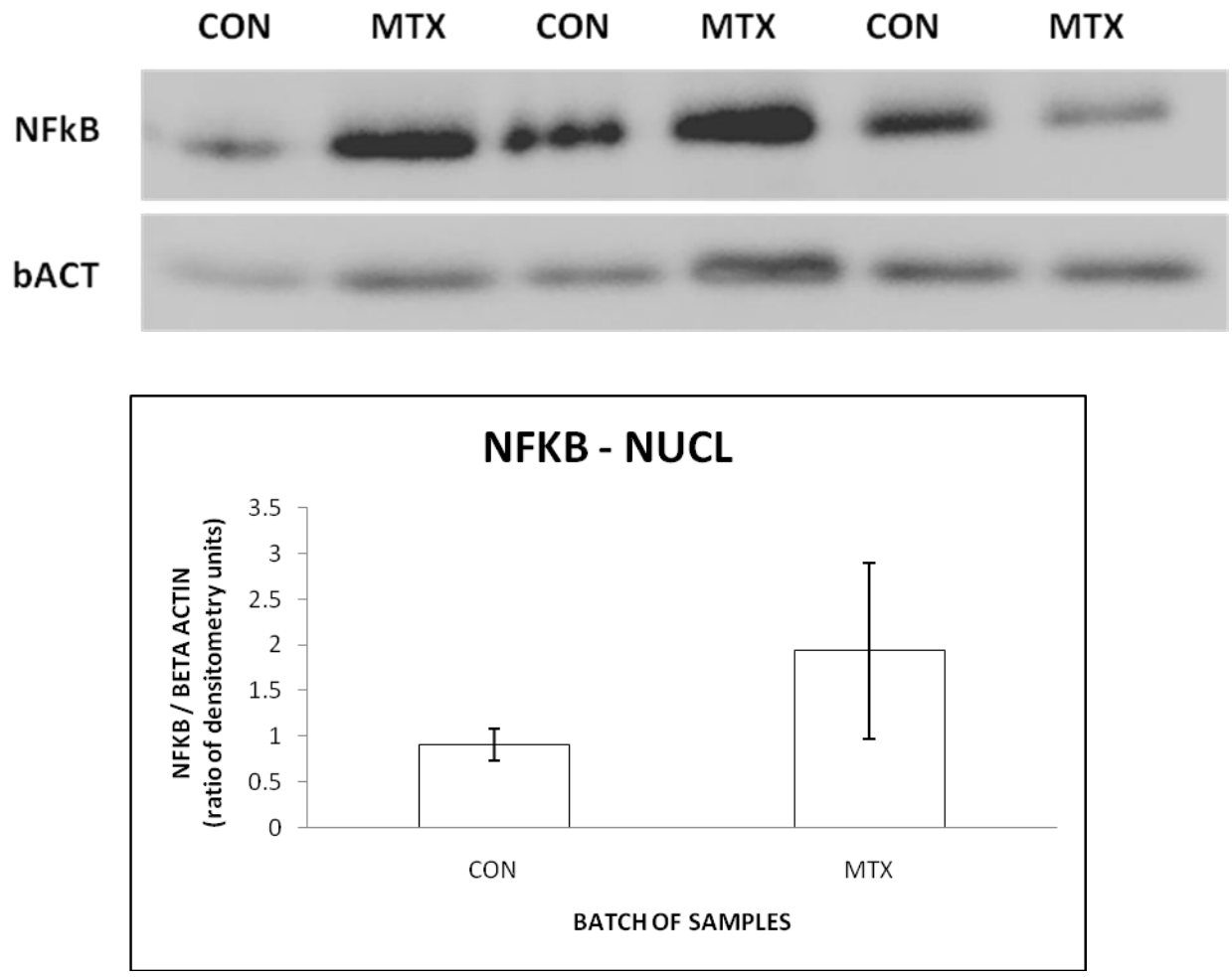
Figure: 6.3.2 **NFκB IHC**



Representative image of nuclear factor kappa B (NFκB) protein expression in the small intestine segments of rats 24 hours after the treatment with vehicle and MTX by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of NFκB when compared to basal immunostaining in control rat small intestine tissue treated with vehicle.

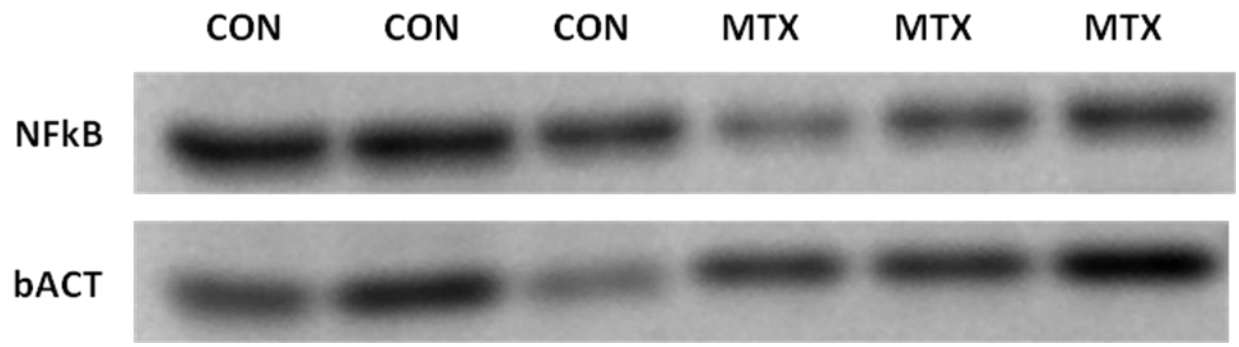
Site	Duodenum	Jejunum	Illeum
VEH	-ve	-ve	-ve
MTX	++	++	++

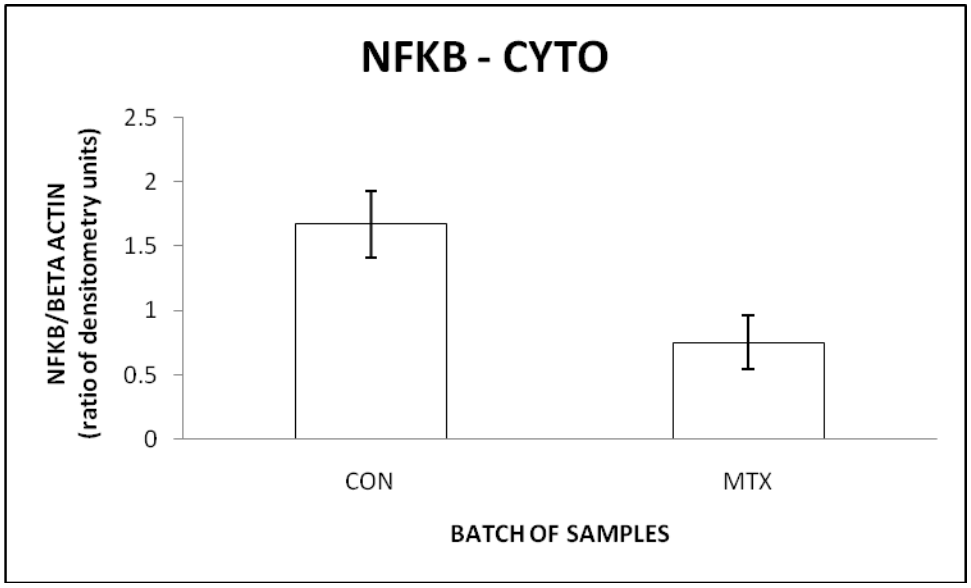
Figure: 6.3.3



Representative blot and corresponding graph of NFκB protein expression in the nucleus fraction of control and MTX treated small intestine mucosa tissue samples after 24hours of final dose. There is increased NFκB expression in nucleus fraction of MTX than control. Data represent mean ± SD, N = 3 in each group.

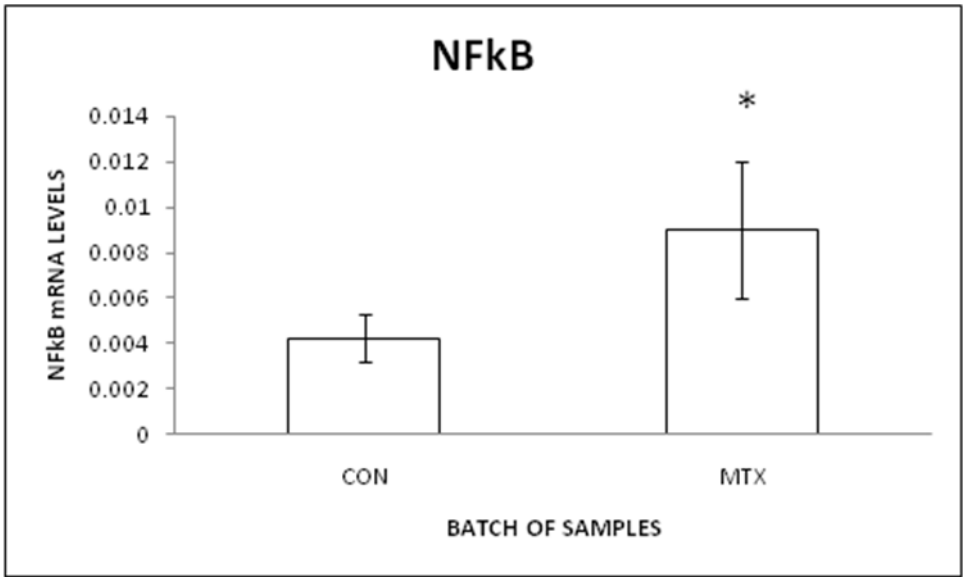
Figure: 6.3.4





Representative blot and corresponding graph of NFκB protein expression in the cytosol fraction of control and MTX treated small intestine mucosa tissue samples after 24hours of final dose. There is decreased NFκB expression in cytosol fraction of MTX than control. Data represent mean ± SD, N = 3 in each group.

Figure: 6.3.5



Effect of MTX on NFκB mRNA levels. RT-PCR analysis of NFκB mRNA expression after 24hours of final dose of MTX and vehicle treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

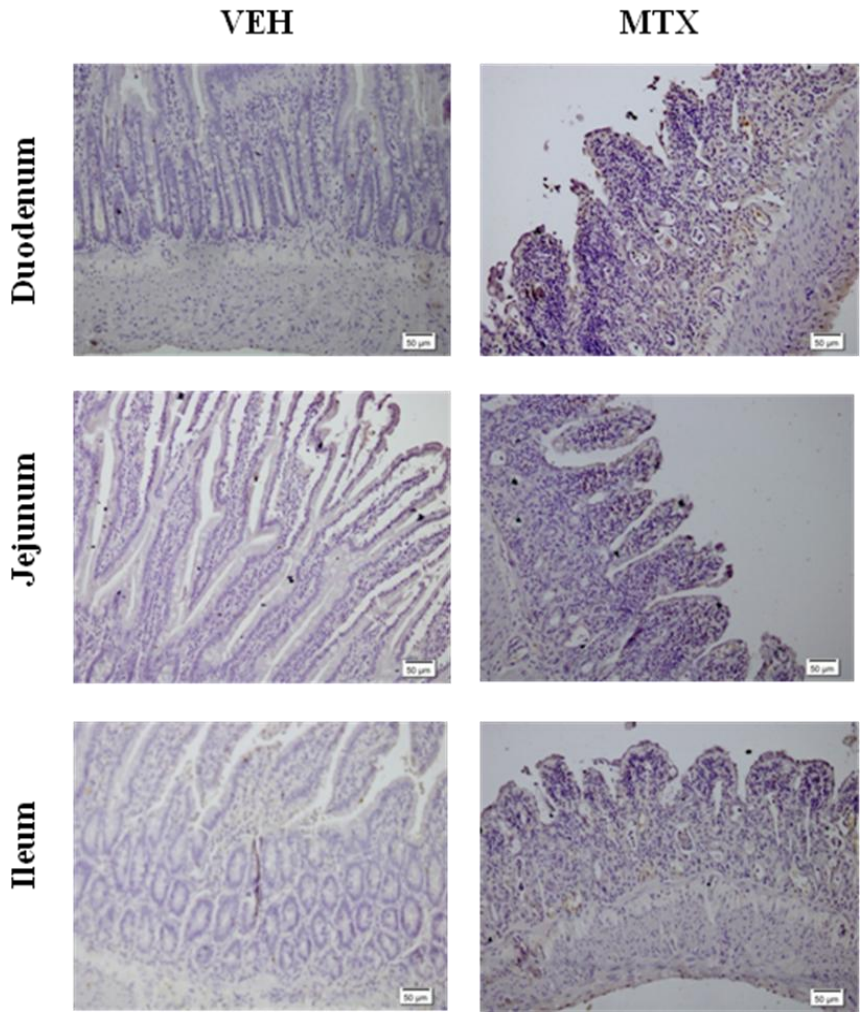
MTX treatment increased protein and mRNA expression of NFκB proinflammatory target genes COX-2, PLA_2 and TNF

Important target genes of NFκB during inflammation are TNF and COX-2, both of which are key mediators in inflammation. Immunohistochemical analysis of control rat intestine showed little COX-2 (Figure: 6.3.6) and PLA_2 (Figure: 6.3.9) staining, whereas after treatment with MTX, COX-2 expression was seen in villus epithelial cells and subepithelial fibroblasts. COX-2 expression was also noted to be present within the cytoplasm of epithelial cells lining the crypts, with expression spanning the entire crypt, and in those lining the luminal surface.

Western blot analysis of intestinal protein levels of COX-2 indicated 2 fold increase (1.59 ± 0.47 vs. 0.43 ± 0.1) in MTX treated rat intestine (Figure: 6.3.7). RT-PCR analysis of COX-2 mRNA expression showed nearly 5 fold (0.0002 ± 0.00008 vs. 0.00003 ± 0.000004, $P = 0.029$) increase in MTX treated rat intestine (Figure: 6.3.8).

Western blot analysis of protein levels of TNF-α in the small intestines of MTX treated rats showed nearly 5 fold (0.53 ± 0.10 vs. 0.12 ± 0.04) increase as compared with control (Figure: 6.3.10). RT-PCR for TNF-α was used as a sensitive biomarker of proinflammatory changes in the intestine. Small intestinal TNF mRNA expression was increased by 2.5 fold (0.0008 ± 0.0001 vs. 0.0003 ± 0.00008, $P = 0.036$) after MTX treatment. (Figure: 6.3.11). These findings suggest a role for inflammatory mediators TNF, COX-2, PLA_2 in MTX mucositis.

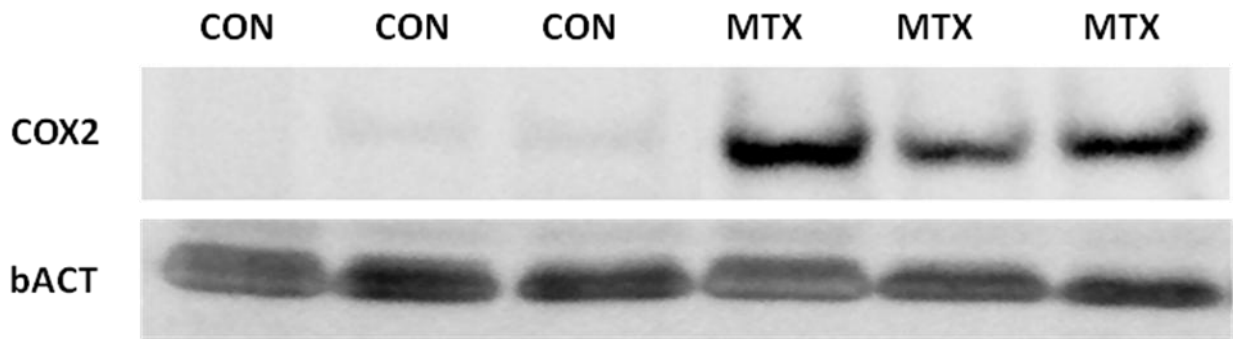
Figure: 6.3.6 **COX2 IHC**

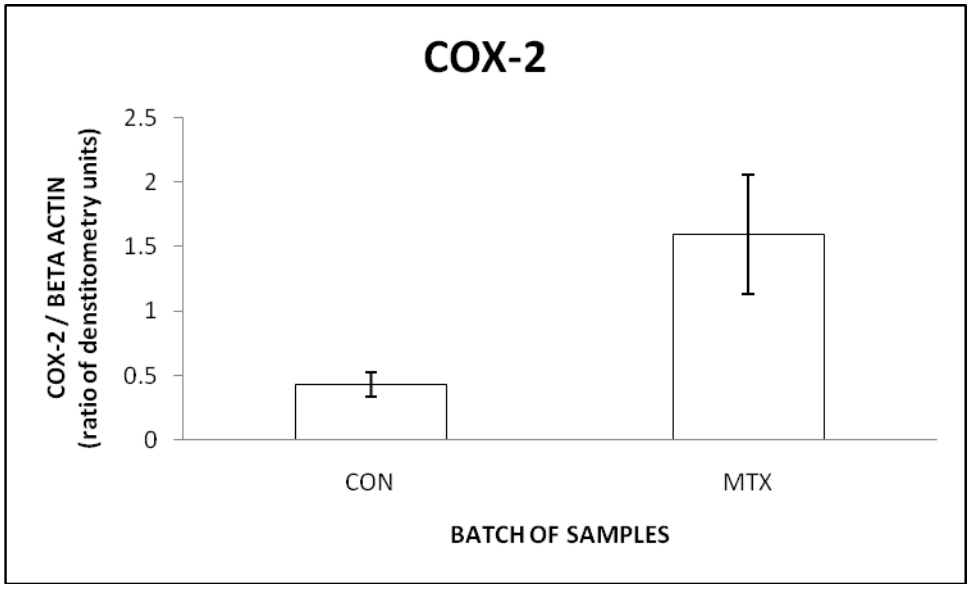


Representative image of cyclooxygenase 2 (COX2) protein expression in the small intestine segments of rats 24 hours after the treatment with vehicle and MTX by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of COX2 when compared to basal immunostaining in control rat small intestine tissue treated with vehicle.

Site	Duodenum	Jejunum	Illeum
VEH	+/-	-ve	-ve
MTX	+	+	++

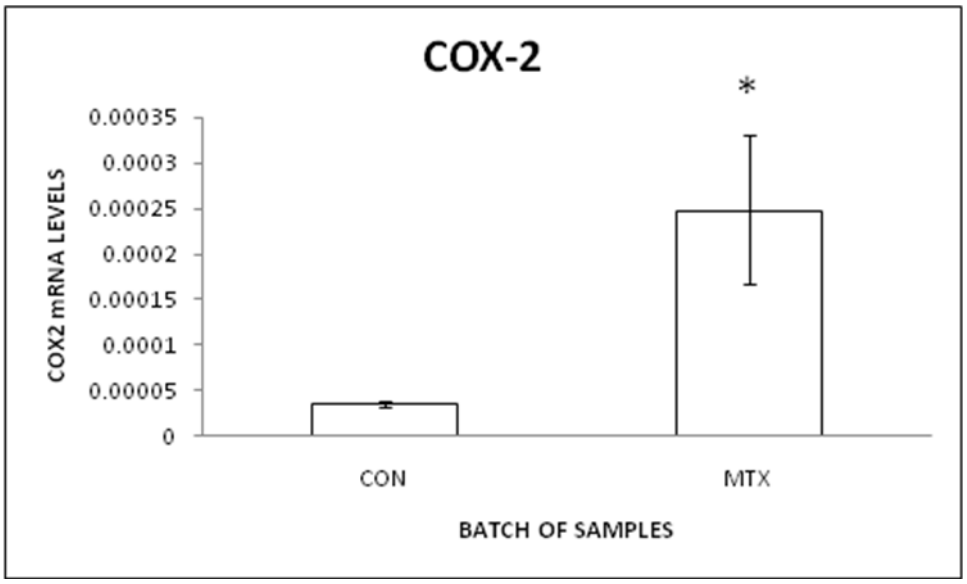
Figure: 6.3.7





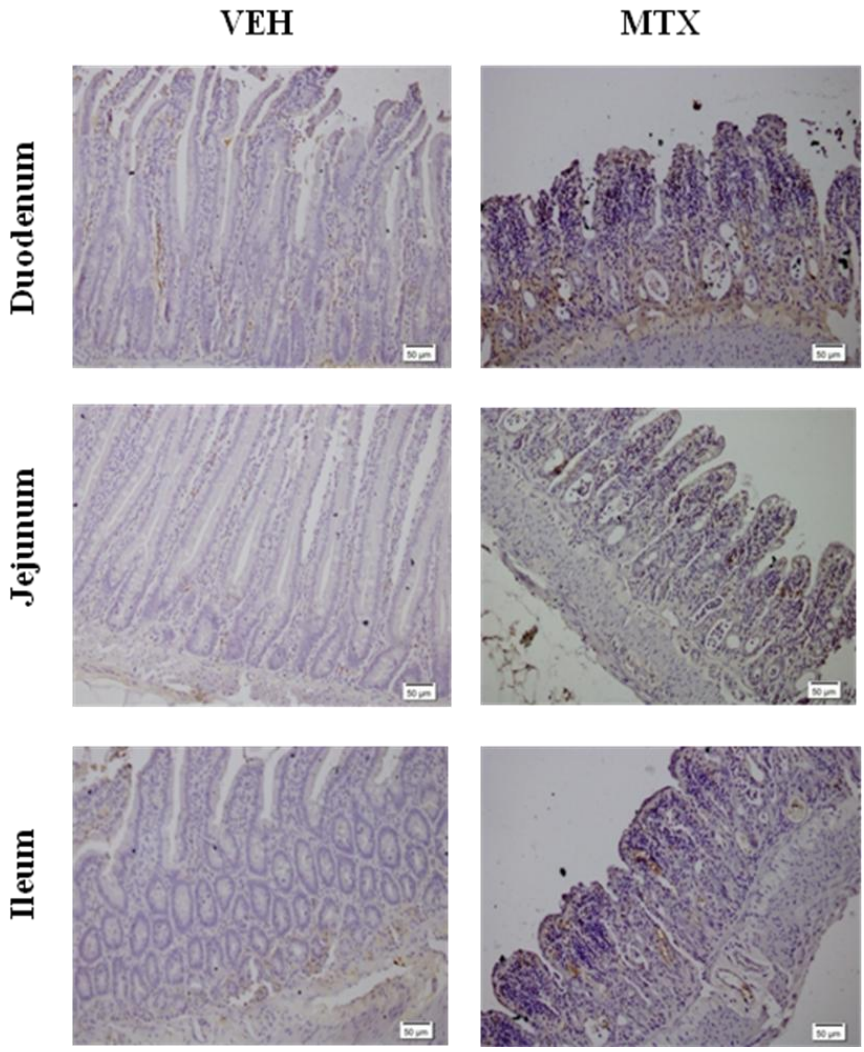
Representative blot and corresponding graph of COX-2 protein expression in the cytosol fraction of control and MTX treated small intestine mucosa tissue samples after 24hours of final dose. There is increased COX-2 expression in cytosol fraction of MTX than control. Data represent mean ± SD, N = 3 in each group.

Figure: 6.3.8



Effect of MTX on COX2 mRNA levels. RT-PCR analysis of COX2 mRNA expression after 24hours of final dose of MTX and vehicle treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

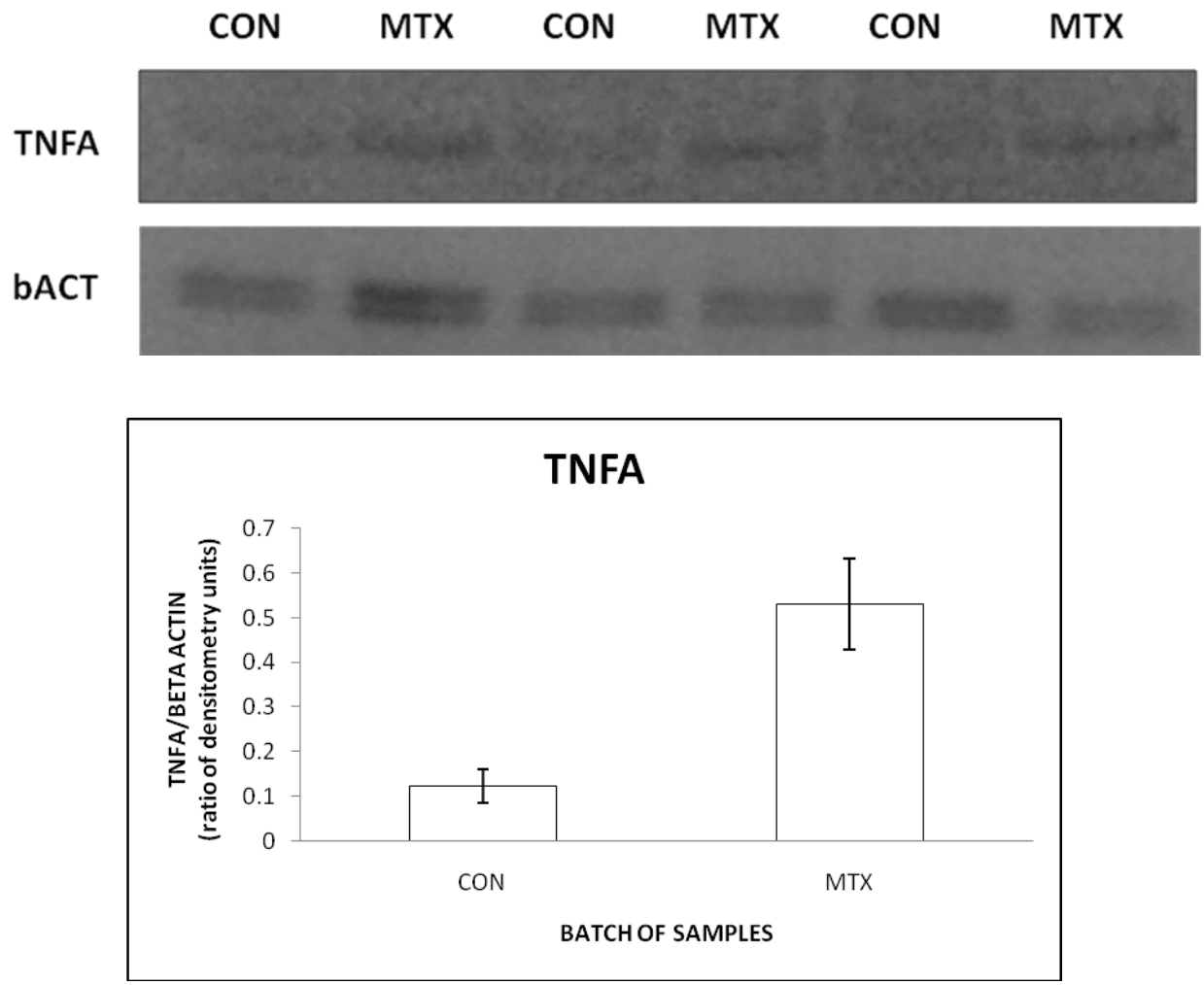
Figure: 6.3.9 **PLA_2 IHC**



Representative image of phospholipase A2 (PLA_2) protein expression in the small intestine segments of rats 24 hours after the treatment with vehicle and MTX by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of PLA_2 when compared to basal immunostaining in control rat small intestine tissue treated with vehicle.

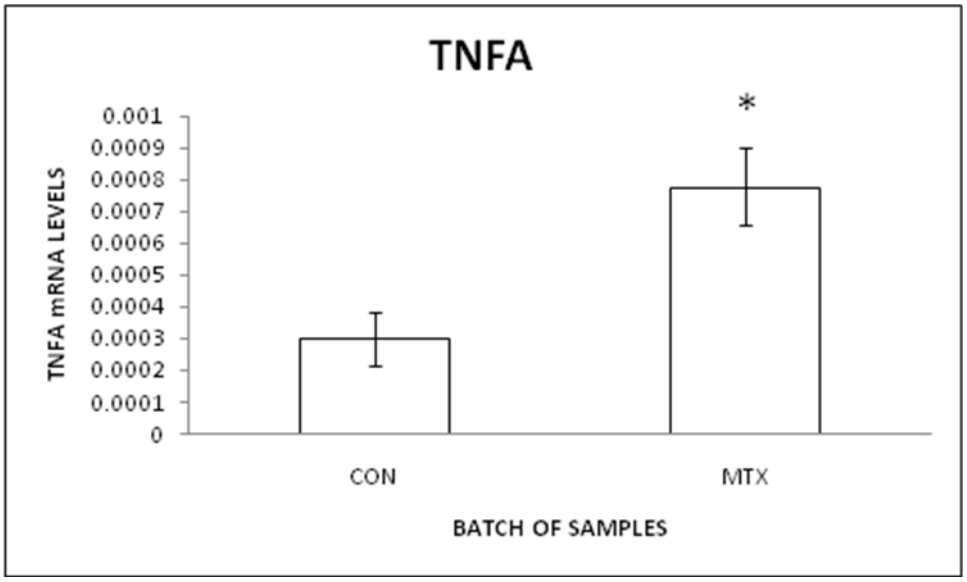
Site	Duodenum	Jejunum	Illeum
VEH	-ve	-ve	-ve
MTX	++	+++	+++

Figure: 6.3.10



Representative blot and corresponding graph of TNFα protein expression in the cytosol fraction of control and MTX treated small intestine mucosa tissue samples after 24hours of final dose. There is increased TNFα expression in cytosol fraction of MTX than control. Data represent mean ± SD, N = 3 in each group.

Figure: 6.3.11



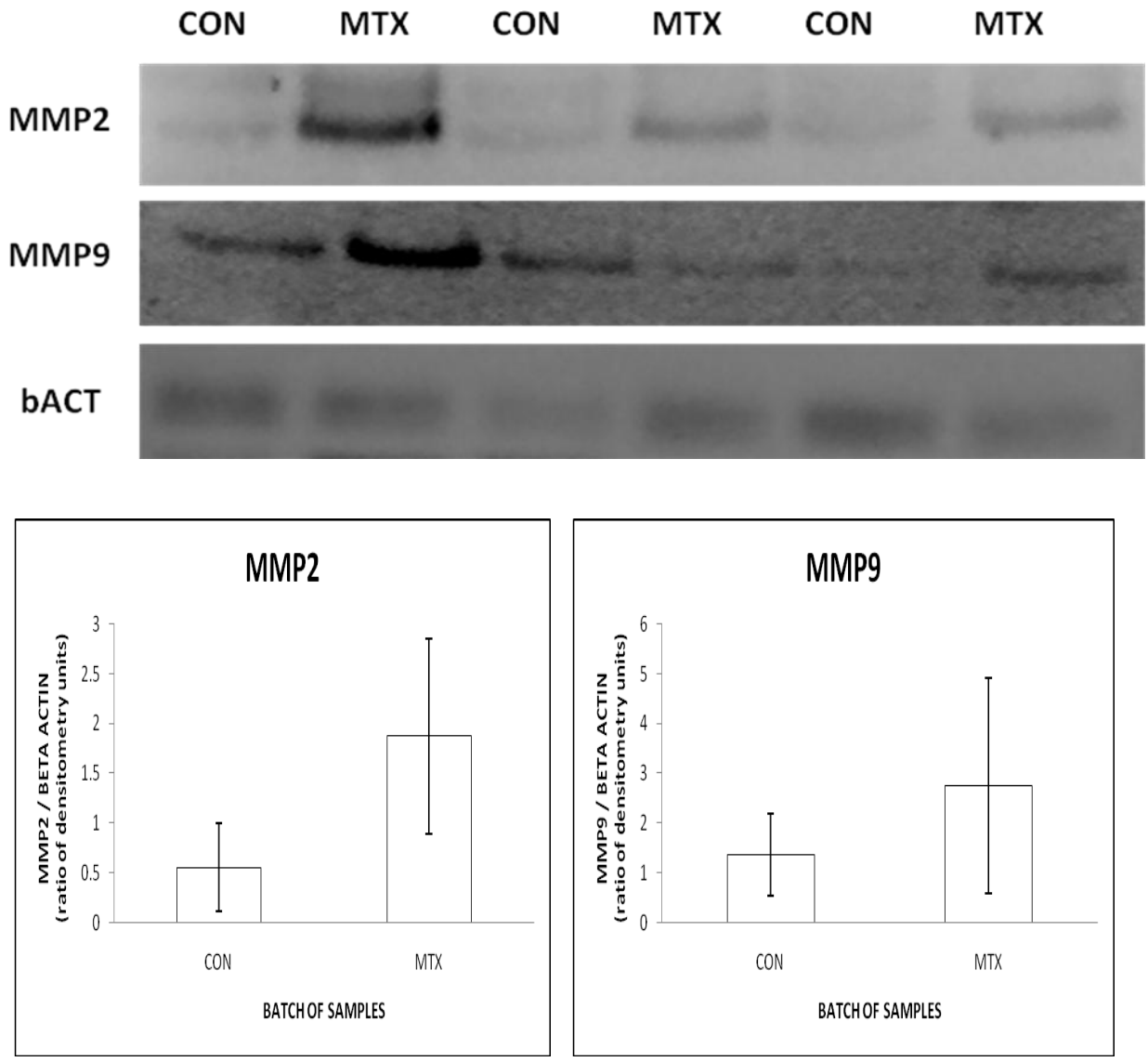
Effect of MTX on TNFα mRNA levels. RT-PCR analysis of TNFα mRNA expression after 24hours of final dose of MTX and vehicle treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

MTX treatment increased protein and mRNA expressions of MMP2 and MMP 9

MMP enzyme family members are responsible for the degradation of connective tissue and the transcription of several MMPs and are mainly regulated by nuclear factor-κB. MMP-9 released from intestinal epithelial cells during inflammation could be responsible for degradation of ECM components with subsequent loss of mucosal integrity. Therefore we next analyzed the protein and mRNA expression of MMPs.

In western blot analysis, MMP-2 (1.87 ± 0.98 vs. 0.55 ± 0.44, P = 0.10) and MMP-9 (2.75 ± 2.15 vs. 1.37 ± 0.8) protein bands were faint in control intestines, but the bands were intense in MTX treated rat intestines (Figure: 6.3.12). In RT-PCR, mRNA expression levels for MMPs 2 and 9 were up-regulated, indicating the transcriptional upregulation of MMPs 2/9. RT-PCR of MMP2 (0.002 ± 0.001 vs. 0.0002 ± 0.00007, P = 0.016) (Figure: 6.3.13) and MMP-9 (0.0004 ± 0.0001 vs. 0.0002 ± 0.00007, P = 0.063) (Figure: 6.3.14).

Figure: 6.3.12



Representative blot and corresponding graph of MMP-2 and MMP-9 protein expression of control and MTX treated small intestine mucosa tissue samples after 24hours of final dose. There is increased MMP2 and MMP9 protein expression in MTX compared to control. Data represent mean ± SD, N = 3 in each group.

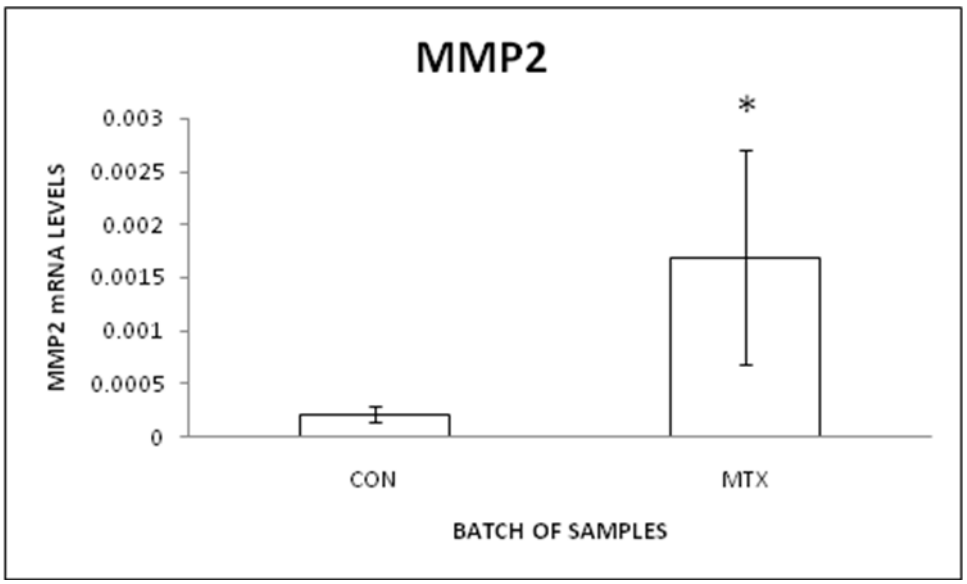


Figure: 6.3.13 - Effect of MTX on MMP2 mRNA levels. RT-PCR analysis of MMP2 mRNA expression after 24hours of final dose of MTX and vehicle treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

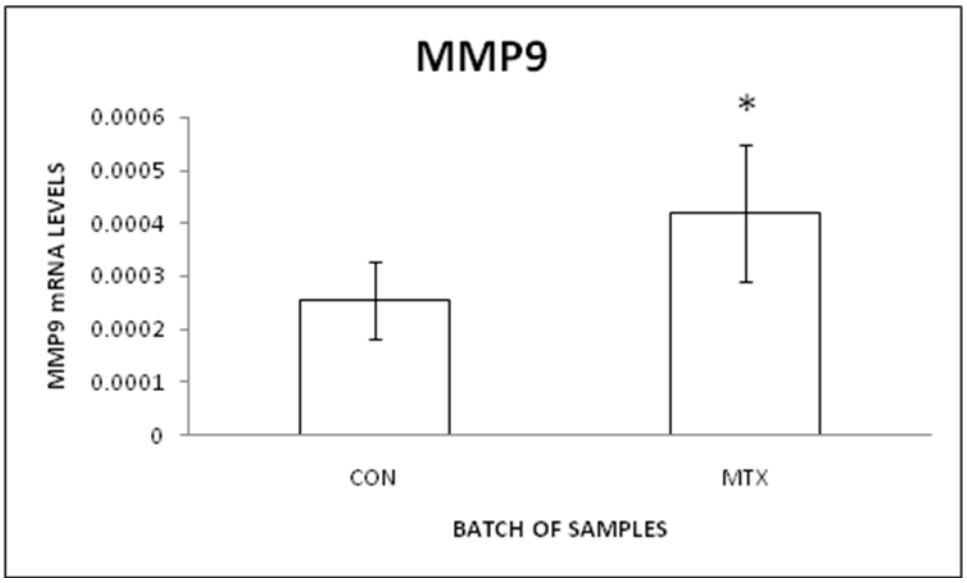
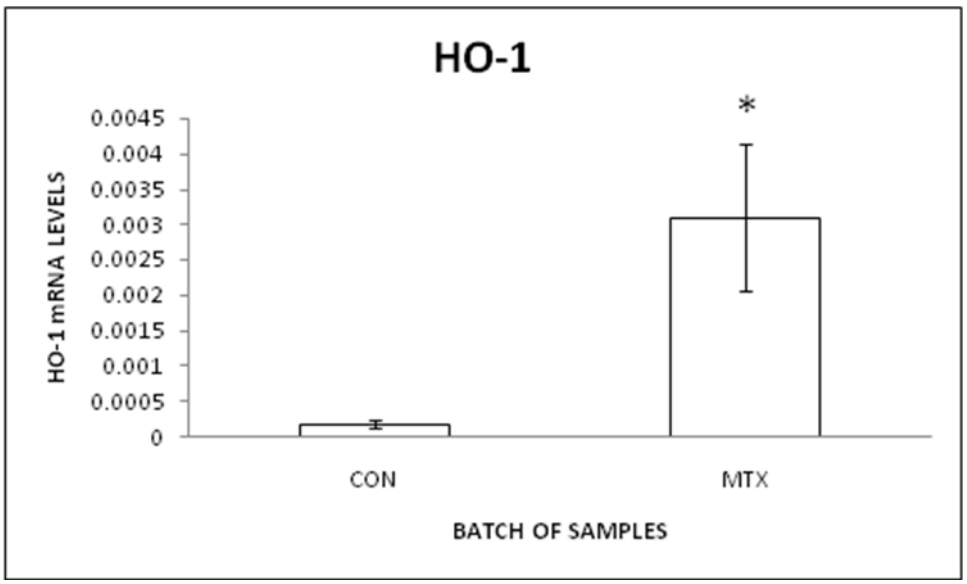


Figure: 6.3.14 - Effect of MTX on MMP9 mRNA levels. RT-PCR analysis of MMP9 mRNA expression after 24hours of final dose of MTX and vehicle treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

MTX treatment upregulates HO-1 and HSP 70 gene expressions

High levels of NO exposure induce protective stress responses, stimulating Hsp70 expression and HO-1 expression. Induction of these HSPs protects cells from damage due to oxidative injury and cytokine-mediated cytotoxicity. The activation of HO-1 could act as a natural defensive mechanism to alleviate inflammation and tissue injury in the gastrointestinal tract. Therefore we assessed the mRNA expressions of HO-1 and HSP70 by RTPCR. We found basal expression of HO-1, which was increased by 18 fold after MTX treatment (0.003 ± 0.001 vs. 0.0001 ± 0.00005, P = 0.010) (Figure: 6.3.15).

HSP 70 was increased by 7 fold (0.0003 ± 0.0001 vs. 0.00005 vs. 0.00001, P = 0.010) (Figure: 6.3.16) in the small intestines of MTX rats as compared to control. This shows that HO-1 and HSP70 are expressed constitutively in normal intestinal mucosa, and are up-regulated in MTX induced inflammation. The up-regulation of HO-1 and HSP70 suggest that their activation could act as a natural defensive mechanism to alleviate intestinal inflammation and tissue injury caused by MTX.



Figure; 6.3.15 - Effect of MTX on HO-1 mRNA levels. RT-PCR analysis of HO-1 mRNA expression after 24hours of final dose of MTX and vehicle treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

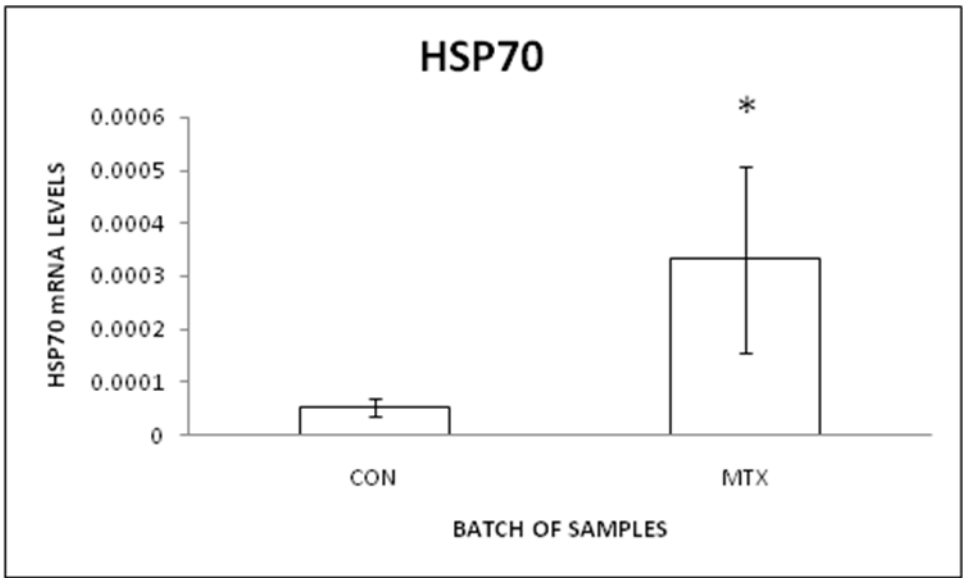


Figure: 6.3.16 - Effect of MTX on HSP70 mRNA levels. RT-PCR analysis of HSP70 mRNA expression after 24hours of final dose of MTX and vehicle treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

6.3.6. DISCUSSION

NF-kappaB has multiple, often opposing functions in the intestine. NF-κB activity is of particular importance for maintenance of epithelial barriers, but it was also proposed that NF-κB activation in epithelial cells can lead to production of inflammatory chemokines that recruit immune cells to the tissue, thereby initiating an inflammatory amplification cascade (133). Antiapoptotic actions of NF-κB in intestinal epithelial cells dominate tissue responses to many acute inflammatory and injurious challenges, whereas proinflammatory and cell survival functions of NF-κB in macrophages, T cells govern chronic intestinal inflammation (134-135).

As the nuclear transcription factor NF-κB is a key regulator of the inducible expression of many genes involved in immune and inflammatory response in the gut we assessed the expression and location of NF-κB p65 was examined by IF and immunohistochemistry in small intestinal tissue. NF-κB p65 protein was detected in nuclei of normal intestinal mucosa, whereas a high expression of nuclear factor appeared in intestinal mucosa of MTX treated rat. Although NF-κB is well characterized as a primary mediator of inflammatory responses during infection and immune reactions, it has recently become evident that NF-κB also mediates a potent cytoprotective, homeostatic function under basal conditions in the intestine, which is challenged not only with a range of microbial pathogens, but is also in constant contact with potent proinflammatory commensal bacteria and their products. Therefore, it is not surprising to find basal intrinsic expression of NFκB in the intestines of normal rats. De. Plaen. etal have showed that NFκB is constitutively expressed in the normal rat intestines (352).

As nuclear translocation of NFκBp65 is often recognized as cell reaction to ROS and cytokine stimulation and correlate with NF-κB-mediated transcriptional activation (353), we measured the level of NF-κB p65 protein in the nucleus and cytosolic fractions of intestinal homogenates using western blot. MTX treatment led nearly a two fold increase in p65 levels in the nucleus as compared with cytosol, suggesting the activation of NFκB pathway after MTX treatment. In addition, using real-time quantitative PCR, we were able to demonstrate nearly 2 fold increase in mRNA expressions of NF-*κ*B p65 in MTX-treated rats as compared with controls. All these findings suggest that MTX induced intestinal inflammation is mediated via activation of NFκB, traslocation into nucleus and increased NFκB gene transcription.

It has long been appreciated that inflammation and infection reduce drug metabolism and that exposure to drug metabolism-inducing xenobiotics can impair immune function. A new study reveals the mutual repression between the xenobiotic nuclear receptor PXR/SXR and NF-kappaB signaling pathways, providing a molecular mechanism linking xenobiotic metabolism and inflammation (354).

Activation of NF-κB is an important event innumerous inflammatory processes of the gastrointestinal tract. Induction of this transcription factor has been reported in Helicobacter pylori gastritis, inflammatory bowel disease, and pancreatitis, rheumatoid synovium, intestinal ischemia and necrotizing enterocolitis and obstruction (133). NF-κB activation is also found in biopsy tissues in cancer patients treated with radiation (144) and several chemotherapeutic drugs, except 5-FU (353). As a consequence of the gene upregulation by the initial activation of NF-κB, a broad range of biological active proteins accumulate and target to the submucosa tissue in the

gastrointestinal tract. NF-κB activation induced by anti-neoplastic agents and radiation is therefore though to elicit the inflammatory and apoptotic responses that lead to the mucosal injury (67, 69)

Once activated by ROS/RNS, NF-κB acts to induce its proinflammatory target gene expression and production of pro-inflammatory cytokines such as , cyclooxygenase-2 (COX-2),tumor necrosis factor (TNF)-α, iNOS, MMP 9,HO-1,interleukin (IL)-1β and IL-6, which in turn lead to tissue injury and apoptosis (345, 355). These cytokines are known to in turn activate NF-*κ*B. The positive feedback is believed to serve to amplify inflammatory signals and exacerbate tissue injury (356).Therefore we next assessed the effect of MTX on protein and gene expression of NFκB target genes involved in inflammation.

In the previous study we have demonstrated increase protein and mRNA expression of iNOS in the small intrstines of rats challenged with MTX. NO is the prototypic redox-signaling molecule more versatile than O_2- or H_2O_2 and clearly better identified with redox-related modifications of intracellular proteins including NFκB. NO and NF-κB signaling pathways are intimately linked. While, NF-κB activation is essential for NOS2 gene transcription (357), and NO regulates NF-κB at various points in its activation cascade (358). *In vivo* studies show that NO up-regulates NF-κB activation in situations like hemorrhagic shock and reperfusion injury. It also has been demonstrated that interfering with iNOS ameliorates hemorrhagic shock- or sepsis-induced NF-κB activation in the gut (359).

Cytokines are key mediators in inflammation, and several cytokines are dysregulated in intestinal inflammation. Important target genes of NFκB during inflammation are TNF and COX-2 both of which are key mediators in inflammation (129, 360). Therefore we analyzed the protein and mRNA expression of these proteins in the small intestines. Significant increase in protein and mRNA expressions of COX-2 and iNOS was observed in the small intestines of MTX treated rats suggesting a role for TNF and COX-2 in MTX induced intestinal injury.

TNF mediates distinct physiological effects through two separate transmembrane receptors, TNFR1, and TNFR2. Physiological levels of TNF result in a preferential ligation to TNFR2, which promotes cellular migration, proliferation, and wound healing, while higher concentrations of TNF lead to ligation of TNFR1 and subsequent activation of inflammatory responses (129, 361). TNF-α up-regulates the transcription of chemokine genes and hence its own synthesis through the activation of NF-κB (362-363). Claud, et al., have previously demonstrated that intraperitoneal TNF-α induces intestinal epithelial NF-κB activation (141). The TNF-α is capable of inducing iNOS expression in various cells. Bertrand *et al*. demonstrated that NSAID induced local production of TNF-α in the small intestine and this event occurred before the elevation of NO production and MPO activity as well as lesion formation (142).

TNF-*α* released early after an inflammatory stimulus, can initiate the infiltration of inflammatory cells into the intestine by activating ICAM-1 and other adhesion molecules (143). TNF is reported to play vital role in various models of GI injury including Radiotherapy-induced sub-acute damage (144) and chemotherapy induced mucositis including MTX , FU and irinotecan. TNF-*α* is a crucial cytokine involved in the pathogenesis of mucositis, which amplifies the

NF-κB signal and initiates mitogen activated protein kinase (MAPK) pathway (145). Studies have shown that methotrexate increased the mRNA levels of TNF-α, IL-1β, MIP-2, and TLR4 in the small intestine (364). 5-FU-induced epithelial damage increased the MPO activity (neutrophil number) and the level of pro-inflammatory cytokines (IL-4, TNF-α, IL-1β and CXCL-8) in the duodenum (365). Infliximab, a monoclonal antibody against TNF-α, led to the reduction of intestinal expression of iNOS in irinotecan treated mice. Thus, suggesting that inflammatory cytokines and nitric oxide are among the main drivers of tissue damage in this model of mucositis (253).

The source of mucosal TNFα is not known at the moment; however, it could be associated to the MTX induced hyperpermeability of the intestinal microcirculation (i.e., TNFα from the circulation could enter mucosa of the small intestine). Alternatively, increase in TNFα levels could be a result of leukocyte recruitment or activation of the intestinal epithelial cells. Regardless of the source of TNFα, increased mucosal levels of TNFα can potentiate the remote intestinal inflammation/injury imposed by MTX. Thus, inflammatory process, once initiated gets amplified continues as a cascade.

The COX enzymes are critical in the biosynthesis of prostanoids, play significant roles in the gut are key for intestinal epithelium maintenance. COX, the enzyme that catalyzes the first two steps in the biosynthesis of the prostaglandins from arachidonic acid, exists in two isoforms. COX-1 is constitutively expressed throughout the GI tract and, at least in the absence of damage or inflammation, is the major source of prostaglandin synthesis in these tissues (151). The inducible form, COX-2, is either undetectable or expressed at extremely low levels in the healthy

GI tract of humans and various animals. However, in response to various proinflammatory stimuli, COX-2 is rapidly induced. Cyclooxygenase-2, a rate-limiting enzyme in the biosynthesis of prostanoids (152), leading to increased prostanoid production. Prostanoids play an important role in intestinal epithelial homeostasis and inflammation (155-156) and are known to protect intestinal cells from apoptosis (153-154).

NF-κB up-regulates the transcription of COX-2, a stress response protein, which in turn leads to the synthesis of prostaglandins involved in the inflammatory cascade resulting in edema and tissue injury (366). NO enhances the activity and expression of COX-2 in a variety of cell types (367). During the inflammatory process, COX-2, is activated over a similar time course to iNOS, and crosstalk between these enzymes was suspected as iNOS-derived NO had been shown to activate COX. During inflammation, iNOS has been shown to interact with, S-nitrosylate, and thus enhance the activity of COX-2. Similarly, iNOS appears to bind to, S-nitrosylate, and increase the activity of phospholipase A2 (PLA_2), an AA-generating enzyme. Thus, NO can promote PG generation via S-nitrosylation of the two key enzymes (PLA_2 and COX-2) involved in PG biosynthesis.

Overactivation of COX-2 and production of PGs have been reported in various conditions of intestinal inflammation such LPS induced GI injury in mice (152), NASID induced enteropathy (155), animal models of necrotizing enterocolitis NEC (156) and inhibition of COX-2 by different agents such as have been shown to inhibit COX-2 , PG production and intestinal inflammation. In both humans and animal model, the role of COX-2 in GI intestinal mucositis is supported by the observations that the administration of COX-2 specific inhibitors prevents GI

injury and inflammation. Patients taking a supra therapeutic dose of rofecoxib or celecoxib had significantly lower rates of GI-related adverse events than those taking a nonselective NSAID (368).

Studies have reported that the role of COX-2 in mucositis may be to increase the severity of mucosal injury and prolong the inflammatory and ulcerative event (70, 366). COX-2 contributes to the activation of matrix metalloproteinases, which then cause the breakdown in collagenous subepithelial matrix by matrix metalloproteinase-1 and destruction of the epithelial basement membrane by matrixmetalloproteinase-3 (14). This breakdown of the mucosal barrier by matrix metalloproteinase-3 would worsen the hypersecretion of chloride and fluid caused by increased prostaglandins (35).

Cytosolic phospholipase A(2)alpha (cPLA(2)alpha) is the rate-limiting key enzyme that cleaves arachidonic acid (AA) from membrane phospholipids for the biosynthesis of eicosanoids, including prostaglandin E(2) (PGE(2)), a key lipid mediator involved in inflammation and carcinogenesis (369). Phospholipase A2 (PLA_2) is a key enzyme in the formation of arachidonic acid metabolites, platelet activating factor, and lysophosphatidylcholine, all of which may take part in inflammatory reactions in the gastrointestinal tract. TNF-α, is known to stimulate $cPLA_2$ (56). Therefore we investigated the effect of MTX on the PLA_2 protein expression by IHC. PLA_2 expression was increased in the small intestines of MTX treated rats, suggesting its role in MTX induced intestinal injury. PLA_2-II has been found by immunohistochemistry in the epithelial cells of the gastrointestinal tract. The enzyme was localised in Paneth cells and in the brush border and cytoplasm of enterocytes (370). The activity of PLA_2 was found to be increased in

the ileal mucosa of patients with Crohn's disease, and the increased PLA_2 activity was associated with early symptomatic recurrent ileal inflammation after surgery (371).

Activation of PLA2-II may have dual effects on the mucosa. It can cause harmful effects on the mucosa by generating free radicals or causing release of lysosomal enzymes (372). On the other hand, PIA2-II might protect the mucosa against invasion of pathogenic microbes or play an immune defensive part mediated by chemotaxis (369). Stduies have shown that cPLA(2)alpha protein is S-nitrosylated, and its activity is enhanced by nitric oxide (NO) and that COX-2 enhances the formation of cPLA(2)alpha-iNOS binding complex. It appears that COX-2-induced augmentation of cPLA(2)alpha S-nitrosylation is mediated at least in part through increased association between iNOS and cPLA(2)alpha. These findings disclose a novel link among cPLA(2)alpha, iNOS, and COX-2, which form a multiprotein complex leading to cPLA(2)alpha S-nitrosylation and activation (148).

In recent years matrix metalloproteinases (MMPs) have been implicated as being key mediators in the development of mucositis, possibly through inflammatory pathways, alterations in extracellular matrix composition, adhesion molecules and tight junctions (176, 373). MMPs can be induced by the activity of pro-inflammatory cytokines such tumor necrosis factor-α, interleukin (IL)-1, IL-6 and IL-8 and NFκB that bind to specific elements on MMP gene promoters (178).

MMP's also regulate inflammation by directly and indirectly acting on pro-inflammatory cytokines, such as TNF-α and TGF-β, to control chemokine activity (374). The MMP family

consists of a group of zinc-dependent enzymes, which can be divided into several subgroups including: collagenases, gelatinases, stromelysins, stromelysin-like matrix metalloproteinases (MMPs), matrilysins, membrane-type MMPs, and other MMPs. They are released as inactive proenzymes and are activated by proteolytic cleavage of the N-terminal domain. Most MMPs are constitutively secreted once they become translated. In gelatinase subfamily of MMPs (MMP-2 and MMP-9), the catalytic domain that includes the Zn^{2+} binding site also contains repeats of fibronectin motifs allowing the ability to bind gelatin, their major substrate. MMPs and their inhibitors (TIMPs) play multiple functions in physiological processes and interact with many other mediators regulating inflammatory processes, cell behavior, and angiogenesis (375).

Studies have shown that MMPs2 and 9 play a role in gut barrier function (177). Therefore we assayed the protein and mRNA expressions of MMPs 2 and 9 in the small intestines of MTX treated rats. We found that MMP-2 and MMP-9 mRNA levels were increased in the mucositis lesions of MTX treated rat intestines as compared to the control intestinal tissues. Moreover, MMPs levels as assessed by Western blotting were elevated in the mucositis lesions.

MMP2 and 9 appear to have apposing effects on the intestines, while MMP 2 has anti-inflammatory effect and MMP9 has proinflammatory effect (179-180). It has been hypothesized that MMP-9 plays an important role in both initiation and termination of the inflammatory response. It is suggested that MMP-2 expression during colitis served to protect from the development of inflammatory response likely through its effect on the epithelial barrier. On the other hand, overexpression of MMP-9 impaired wound healing in cultured intestinal epithelial cells in vitro and modulated colonic epithelial differentiation, suggesting that MMP-9 mediates

inflammatory response and/or tissue damage. It is implicated that the tissue damaging effect of MMP-9 overrides the barrier protective role of MMP-2 during colitis (179).

MMP-9, the largest and most complex member of the MMP family, regulates a variety of cellular functions, including proliferation, differentiation, and angiogenesis. Endogenous tissue inhibitors of MMPs (TIMPs) regulate their activation, and TIMP-1 shows greater preference for MMP-9 than any other MMP. Activation of proMMP-9can also be mediated by MMP-2 (376). MMP-9 is released by epithelial cells and neutrophils and is a key effector molecule of inflammatory cells, aiding migration of inflammatory leukocytes through tissue barriers, lysing protein substrates, modulating smooth muscle cell migration, and promoting angiogenesis (373). Castaneda et al have demonstrated that epithelial, but not immune cell derived, MMP9 mediates inflammation (377). In addition to its role in regulating inflammation, MMP9 also plays an important role in epithelial cell differentiation by mediating the preferential differentiation of progenitor cells to enterocytes (179).

MMP-9 plays an important role in the development of gastric damage induced by an NSAID in rats. In a rat experimental model, up-regulation of MMP-9 induced intestinal barrier dysfunction and bacterial translocation (378). Furthermore, the administration of MMP inhibitor ONO-4817 significantly attenuated not only MMP-9 activity but also indomethacin-induced small intestinal damage. ONO-4817 specifically inhibited MMP-2 and MMP-9, but not other MMPs. a chymase inhibitor, TY-51469, prevents the development of small intestinal damage may depend on the inhibition of MMP-9 activation in small intestinal damage after indomethacin administration. Studies have demonstrated that genetic ablation of MMP9 protected mice from chemical (DSS-

and TNBS-) as well as bacteria-(Salmonella)-induced colitis (379-380). Several studies have shown that MMP-2 and MMP-9 are highly expressed in IBD inflamed colonic mucosa of IBD patients and are associated with disease activity (381). MMPs have been shown to function in several of the pathways which are known to be up-regulated in mucositis and contribute to tissue injury and inflammation in many pathological conditions including chemotherapy and indomethacin (177, 382).

Expression of heat shock protein (HSP) is an endogenous mechanism by which living cells adapt to stress. Induction of HSPs protects cells not only from damage due to heat but also from damage due to oxidative injury and cytokine-mediated cytotoxicity (157). Interestingly, these same oxygen-free radicals, such as superoxide, also induce the expression of various HSPs which take part in the defense against oxidative stress. HSPs are expressed in many tissues, including heart, brain, muscle, lung, kidney, liver and intestinal and colonic epithelium (157). These highly conserved molecules are responsible for maintaining adequate protein folding (383) and influencing the degradation of proteins (384) and cell repair processes after damage (158). Furthermore, HSPs are involved in the modulation of immune responses, autoimmunity, cell signalling, cell proliferation, apoptosis, and tumour cell differentiation and invasion (157).

Based on their molecular weight they can be classified into six major families: small HSPs (molecular weight <30 kDa), HSP60s, HSP70s, HSP90s, HSP100s, and other non-ubiquitous HSPs. HSPs can act as “danger signals” for the immune systematic sites of tissue injury (159). HSP70 can also promote cell survival by inhibiting the mitochondrial translocation of Bax and subsequent release of cytochrome c and activation of caspase-9 and -3 (385), an intrinsic

apoptotic pathway that is initiated by intracellular stress signals (160). Induction of Hsp70 protects cells not only from damage due to apoptosis induction but also from damage due to oxidative injury. Furthermore, only cells overexpressing Hsp70 were found to be protected from both ROI- and TNF induced cytotoxicity.

High levels of NO exposure induce protective stress responses, stimulating Hsp70 expression (161). NO donor-induced Hsp70 expression inhibits both ROI production and lipid peroxidation. Accumulated data suggest that relevant levels of nitric oxide may contribute to apoptotic pathway suppression by the upregulation of Hsp70 and that interaction is an early line of defense for protecting cells from death. Heat shock proteins (HSPs) play an important role in mucosal defense of gastrointestinal tract (163, 386). Since MTX induced small intestinal injury is associated with overproduction of NO and ROS, induction of iNOS and NFκB activation, we investigated the levels of hsp 70 expression in the small intestines of rats after MTX treatment. MTX treatment resulted in significant upregulation of hsp 70 in the small intestines. The upregulation of hsp70 may be a defense mechanism against MTX induced overproduction of ROS and NO. Studies have shown that the expression of HSPs in the small intestine is normally negligible (162), but the expression of HSP25 and HSP70 is markedly increased in the intestinal epithelial cells under stress (163). The predominant localization of HSPs in intestinal epithelial cells suggests their primary role is in maintaining the integrity of the enterocyte layer, as demonstrated by Kojima *et al* (164).

HSP 70 protects cells against stress by two mechanisms. Firstly, HSP70 activates antioxidant enzyme activities (such as superoxide dismutase, glutathione peroxidase, and total antioxidant

capacity) and inhibits lipid peroxidation. Secondly, HSP70 reduces iNOS expression by specifically binding to iNOS and its transcription factor Krueppel-like factor 6; moreover, its upregulation was shown to inhibit nuclear factor (NFκB) activation, thereby providing cellular protection against stress (387). HSP70s protect intestinal epithelial cells by preserving the integrity of the actin cytoskeleton and cell-cell contact (163, 388).

Heme Oxygenase (HO)-1, known as HSP 32, is ubiquitously distributed in mammalian cells and tissues and is potently induced by various stimuli, such as oxidative stress and pathological conditions (26). Heme oxygenase-1 (HO-1) is the inducible of three isoforms of HO, the enzyme that catalyzes heme breakdown, generating equimolar amounts of bilirubin, free iron, and carbon monoxide (CO) (165). Many studies showed that HO-1 is involved in a variety of regulatory and protective cellular mechanisms as a stress-responsive protein (389). Heme oxygenase (HO) 1 is inducible by a variety of oxidative stress and is thought to play an important role in the protection of tissues from oxidative injuries. In addition, HO-1 is also reported to be one of the target of NFκB which is plays a pivotal role in inflammation. It is important to mention that HO-1 gene expression is not only regulated via NF-κB 1 but that HO-1 can modulate the activity of NF-κB in various cell types (166).

As MTX induced intestinal injury is associated with increased c oxidative stress and NFκB activation, we were interested in finding out whether threes ia nay alteration in HO-1 induction in the small intestines of rats after MTX treatment. There was basal expression of HO-1 in the normal rat intestines, and after MTX treatment there was a massive increase (nearly 15 fold) in HO-1 mRNA. This may be an adaptive defense mechanism in order to combat MTX induced

Oxidative stress. Studies have shown that HO-1 is expressed constitutively in normal gastrointestinal tract (GIT) (390-391). The GIT is lined by a simple epithelium that separates the hostile processes of digestion and absorption that occur in the intestinal lumen from the aseptic environment of the internal milieu by defensive mechanisms. GIT undergoes constant oxidative stress, inflammation and cell cycle/apoptosis. The normal expression and up-regulation of HO-1 after MTX treatment indicate that activation of HO-1 could act as a natural defensive mechanism to alleviate inflammation and tissue injury caused by MTX These findings show that HO-1 induction may plays a fundamental role in protecting mucosal cells of the intestine from oxidative damages induced by MTX and also that the activation of HO-1 could act as a natural defensive mechanism to alleviate inflammation and tissue injury in the gastrointestinal tract.

HO-1 is expressed constitutively in normal gastric, intestinal, and colonic mucosa (167), and is up-regulated in their inflamed tissue (168). HO-1 induction plays a fundamental role in protecting mucosal cells of the intestine from oxidative damages (169). Chung and coworkers (170) demonstrated that there is an exaggerated lethality in HO-1 homozygous knockout mice (KO, *Hmox*1$^{-/-}$) following sepsis due to gross tissue destruction and loss of bowel integrity in the ileum and colon. Overexpression of HO-1 in smooth muscle cells and myofibroblasts of blood vessels was shown to increase bacterial clearance by enhancing phagocytosis and the endogenous antimicrobial response, thus improving survival.

Interestingly, expression of HO-1 is usually increased in gastrointestinal inflammation and injury. This was shown in gastric ulcers (392), colitis (393), radiation enteritis (171), inflammatory bowel disease (IBD)(172) of animal models or patients suggesting that the activation

of HO-1 may act as an endogenous defensive mechanism to reduce inflammation and tissue injury in the intestinal tract.

Several agents reportedly exhibit cytoprotective action in the gastrointestinal mucosa through induction of HO-1. Moreover, some agents including glutamine, tranilast, RDP58, Octreotide, lansoprazole, Ketamine, Polaprezinc (PZ, an anti-ulcer drug) and gliotoxin may contribute to the preservation of gastrointestinal mucosa in some experimental models, such as colitis, radiation enteritis, and acute gastric mucosal lesions his protective effect is partly mediated by the induction of HO-1 expression. The administration with a HO inducer (hemin) results in lessened mucosal injury and improved intestinal transit following gut I/R 374(394). These results demonstrate that HO-1 may be implicated in cytoprotection and may be an effective agent for the treatment of diseases characterized by mucosal inflammation in GIT.

The protective role of HO-1 appears to be mediated by the anti-inflammatory and immunomodulatory effects of its products, CO, biliverdin, and bilirubin (395). HO-1 detoxifies the pro-oxidant heme into CO, Fe and biliverdin. The latter is subsequently converted into bilirubin, a potent endogenous free radical scavenger and antioxidant. In addition, the HO-1product CO has been reported to have anti-inflammatory and anti-apoptotic effects immunomodulatory properties (396).

In this study, we found that NF-κB inflammatory pathway was induced by MTX in the intestine as evidenced by upregulation of its target proinflammatory genes, iNOS, COX-2, TNF, MMP and PLA_2. Studies have shown that activation of NFκB and subsequent upregulation of

proinflammatory cytokines may be a potentially important factor in the pathobiology of chemotherapy induced by irinotecan, methotrexate (MTX) (60, 67). However, in 5-FU-induced mucositis in rats, the role of NF-κB remains controversial. Logan etal (344) have done Immunochemistry on mucosal tissue of rats treated with 5FU showed elevation of TNF-α and IL-1β levels but no significant increased staining for NF-κB and IL-6. These authors have suggested that apoptotic and inflammatory changes in 5-FU-induced mucositis may be secondary to pathways independent of NF-κB. Interestingly, NF-κB activation is also found in biopsy tissues in cancer patients treated with radiation and several chemotherapeutic drugs, except 5-FU (67, 69). However, recent studies (397-398) suggest a role for NFκB in FU mucositis. Thus, pathobiology of chemotherapy-induced alimentary tract mucositis appears to be influenced by the type of mucotoxic drug administered. Therapeutics targeting NFκB inhibition may help in confirming the central role of this key driver of inflammation in MTX induced enteritis.

STUDY – IV

Role of apoptotic pathway in methotrexate induced small intestinal injury

STUDY IV: To investigate the effect of MTX on the apoptotic pathway in the small intestines

6.4.1 ABSTRACT

Protein tyrosine nitration of proteins modifies functions and activities of such proteins. Many studies have shown that such protein modifications have consequences like alteration in biological activity, increase/decrease in its expressions, alterations in biochemical reactions leading to changes in the cellular functions. Apoptosis is one of mechanism which occurs due to the changes in the protein involved in the process. It has been shown that nitration of cytochrome-c leads to the release from mitochondria to cytosol and the activation of the caspase cascade. The hypothesis of this study is to detect the role of intrinsic apoptotic pathway in MTX induced small intestinal injury. To study this, rats were treated with MTX 7mg/kg body weight and control rats with vehicle alone. All the rats were sacrificed after 24 hours of the final dose of MTX and the small intestine was removed, washed with saline and the mucosal scrapings was collected and stored. A piece of tissue was used for the process of immunohistochemistry and immunofluorescence of proteins involved in the pathway. The cell fractions was isolated from the mucosa by ultracentrifugation method and utilized for western blots of cytochrome-c, caspase-3, caspase-9 and parp. Some of the mucosa sample was immediately processed for DNA isolation to assess the fragmentation. We observed a significant release of cytochrome-c from 2.2 fold decrease in mitochondrial fraction to 2 fold increase in cytosol in the MTX treated rats when compared to control. The expression of cleaved fragments of caspase-9, caspase-3 and PARP was significantly increased by 2.8 fold, 4 fold, 1 fold in MTX treated rats. The caspase 3 gene expression decreased by 80% and caspase 3 activity decreased by 50% in MTX treated rats.

The expression of PARP in the nucleus was increased in the immunofluorescence staining of MTX segments. We also found increased DNA fragmentation and decreased DNA content in MTX treated rats when compared to control. In conclusion, the study shows that the intrinsic apoptotic pathway is activated in MTX treated rat intestines.

6.4.2 INTRODUCTION

Exposure of cells to peroxynitrite leads to cell death via two possible mechanisms, necrosis or apoptosis. Cell death primarily through inhibition of mitochondrial respiration and loss of ATP production is normally classified as necrotic. By contrast, apoptotic cell death is characterized by the ATP-dependent activation of cysteine proteases (caspases) (182). Recently, a large number of studies have associated mitochondrial dysfunction caused by ROS/RNS lead to both accidental cell death (necrosis) and programmed cell death (apoptosis) (184-186).

Peroxynitrite can trigger the release of mitochondrial pro-apoptotic factors and trigger cytochrome-c-dependent apoptosis in the cytosol through the peroxynitrite dependent oxidation of permeability transition pore components (108, 399). Mitochondrial outer membrane permeabilization (MOMP) leads to the release of cytochrome c (cyt c) into the cytosol. Cyt c is a key activator of caspases. Although peroxynitrite may cause necrosis by de-energizing the mitochondria, it can also lead to apoptotic cell death via activation of the caspase machinery MOMP and the resulting efflux of proapoptotic signaling molecules occur universally following peroxynitrite exposure. Cytosolic cyt c acts in concert with apoptosis activating factor-1 (Apaf-1) to activate procaspase9. Active caspase 9 is an initiator caspase, which in turn activates

effector caspases, such as caspases 3 and 7. Active effector caspases execute apoptosis by dismantling key proteins and cellular structures (187).

Caspase-3, one of the key executioners of apoptosis, is essential for DNA fragmentation and the morphological changes associated with apoptosis. Caspase mediated apoptotic cell death is accomplished through the cleavage of several key proteins required for cellular functioning and survival. PARP-1 is one of several known cellular substrates of caspases (400).Cleavage of PARP-1 by caspases is considered to be a hallmark of apoptosis. The cleavage of PARP-1 by these caspases results in the formation of 2 specific fragments: an 89-kD catalytic fragment and a 24-kD DBD (401-402). Under basal conditions, the primary function of PARP-1 is to detect and repair DNA damage. However, cells with severely damaged DNA have amplified PARP-1 activity resulting in high NAD+ consumption (depleting ATP pools). If unchecked, this activity inevitably leads to passive necrotic cell death (resulting from prolonged ATP depletion) (403). This process is blocked by rapid cleavage and inactivation of PARP-1 by the action of caspases. However, insults which initiate necrosis cause PARP-1 overactivation that proceeds unchecked due to inadequate caspase activation, lower PARP-1 cleavage and less PARP-1 24-kD fragment formation (404).

Apoptosis is an inherent protective mechanism used by cells to regulate proliferation and occurs at a low level in the healthy, normal small intestine. Thus, apoptosis is an important factor in gastrointestinal physiological cell renewal, which can be triggered by noxious stimuli such as trauma and ischemia (205-206). Previous studies definitely demonstrated that apoptosis of the intestinal epithelial cells plays an important role in the gut barrier damage and increased

permeability of the intestinal epithelium, leading to possible translocation of intraluminal microbes and bacterial toxins (143, 207). Previous studies using irinotecan, 5FU, MTX have shown that the earliest effect of chemotherapy is the rise of apoptosis in intestinal crypts (58, 64, 405).

Apoptosis is considered playing a critical role in the occurrence of intestinal mucositis induced by 5-FU chemotherapy (406). In fact, a large number of apoptotic cells where found in mouse intestinal crypt prior to serious mucosal destruction and in intestinal biopsy specimens of patients undergoing chemotherapy (58). The mechanisms involved in chemotherapy-mediated induction of small intestinal cell apoptosis are still poorly understood. Therefore, in the present study we investigated in detail the role of apoptotic pathway in MTX induced mucositis. Apoptosis was evaluated by (a) Cytochrome c release from mitochondria to cytosol, (b) IHC detection of active caspases -9 and 3 and PARP-1, (c) Immunoblot detection of caspases -9 and 3 and full length and cleaved PARP-1, (d) Quantification of DNA fragmentation and (e) Hematoxylin and Eosin (H&E) staining

6.4.3 AIM and OBJECTIVES

The present study was designed to investigate the apoptotic pathway in MTX treated rat intestines.

The objectives of the present study is to assess the apoptotic pathway by the

- Quantification of DNA fragmentation by laddering
- Immunoblot of cytochrome c in the cytosol and mitochondria (to determine cyt c release)

- Immunoblot detection of active caspase 9, caspase-3 and cleaved PARP
- IHC detection of caspases -9 and 3 and PARP-1
- Assay of caspase 3 activity by ELISA and mRNA by RTPCR
- Hematoxylin and Eosin (H&E) staining for apoptotic cells

6.4.4 SUMMARY OF EXPERIMENT

Adult male wistar rats (200 – 250g) were treated with MTX 7mg/kg body weight and the control rats were treated with vehicle alone intraperitoneally for three consecutive days. After 24 hours of the final dose of MTX the rats were sacrificed and the entire length of small intestine tissue was removed, washed with saline and a piece of segments (duodenum, jejunum, ileum) was stored for immunohistochemistry studies. The mucosa scrapings was collected and used for the isolation of cell fractions by ultracentrifugation and also for the isolation of DNA. The cell fractions was used for the western blot of cytochrome-c, cleaved caspase-3, caspase-9 and PARP. The DNA isolated was used for DNA fragmentation assay.

6.4.5 RESULTS

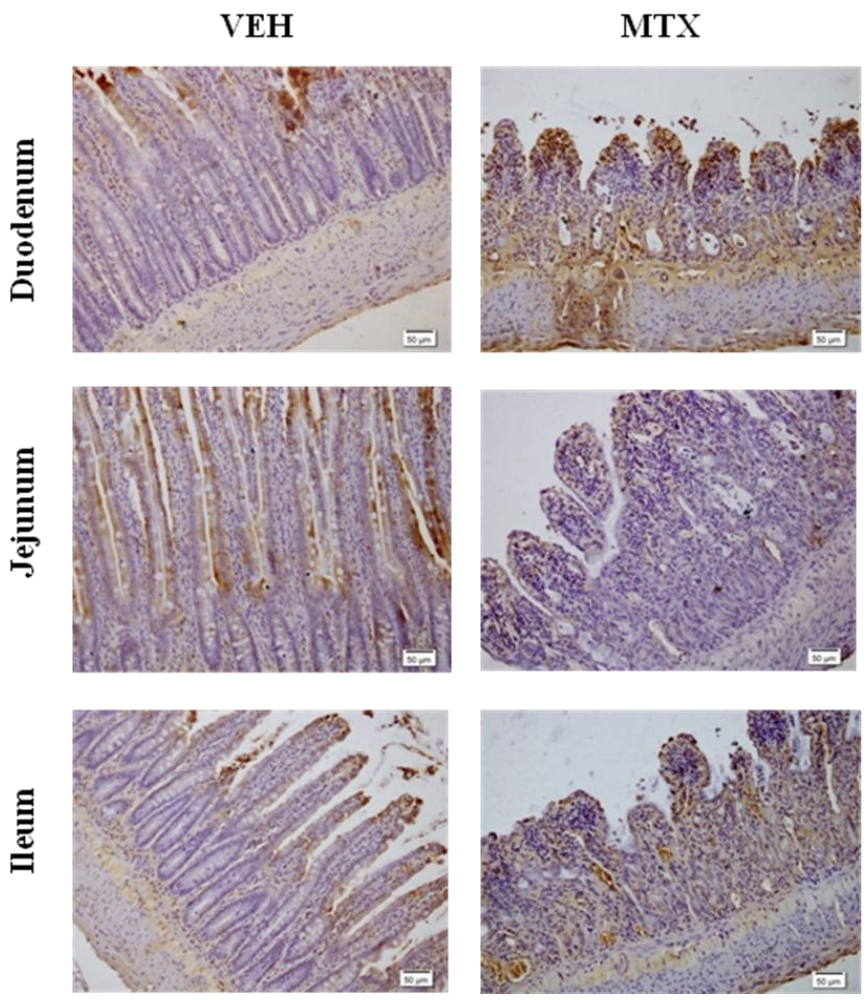
MTX treatment resulted in cytochrome c release into the cytosol

Cytochrome c is released from mitochondria into cytosol through the MPT pore during an early period of apoptosis. To evaluate the degree of cytochrome *c* release after MTX-treatment immunocytochemical staining and Western blot analysis using anti-cyt c antibody.

Immunolocalization of cytochrome c (CYTC) (Figure: 6.4.1), protein expression revealed increased expression in enterocyte regions, lamina propria cells of duodenum, jejunum and ileum of MTX as compared to control. Weak and uniform basal immunostaining was found in the normal control rats small intestine segments which treated with the vehicle. As shown in Figure: 6.4.1, mitochondria-enriched perinuclear regions of control rat intestines cells stained clearly with the antibody. However, perinuclear localization of cytochrome *c* decreased markedly after MTX treatment.

Western blot was used for analyzing cytochrome *c* release using anti-cytochrome *c* antibody in cytosolic fraction and mitochondria. In the control rat intestine cyt c was mainly present in the mitochondrial fraction, and cytosol had negligible amounts. In the MTX treated rat intestinal homogenates, cytochrome *c* level accumulated in the cytosolic fraction, suggesting cyt c release after MTX treatment. Western blots revealed cytochrome c expression decreased by 2.2 fold (0.319 ± 0.2 vs. 2.49 ± 3.1) in mitochondria fraction and 2.0 fold (2.26 ± 2.0 vs. 0.002 ± 0.003) increased in cytosol fraction of MTX treated samples (Figure: 6.4.2).

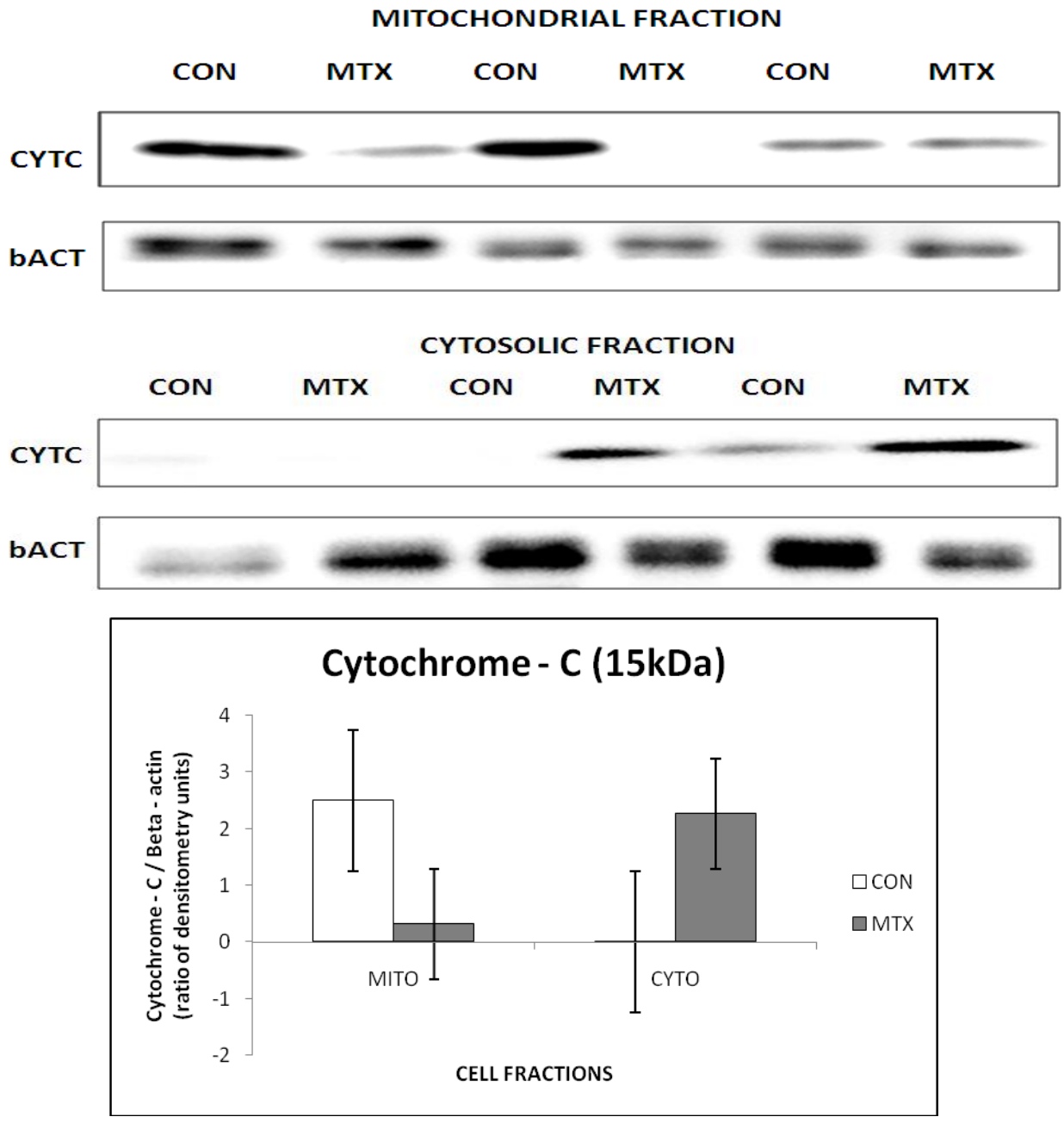
Figure: 6.4.1 **CYT-C IHC**



Representative image of cytochrome c (CYTC) protein expression in the small intestine segments of rats 24 hours after the treatment with vehicle and MTX by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of CYTC when compared to basal immunostaining in control rat small intestine tissue treated with vehicle.

Site	Duodenum	Jejunum	Illeum
VEH	+/-	+/-	+/-
MTX	+++	++	+++

Figure: 6.4.2



Representative blot and graph of CYT-C protein in mitochondria and cytosol fraction of control and MTX treated small intestine mucosa tissue samples after 24hours of final dose. There is decreased CYT-C in mitochondria fraction and increased in cytosol fraction of MTX as compared to control. Data represent mean ± SD, N = 3 in each group.

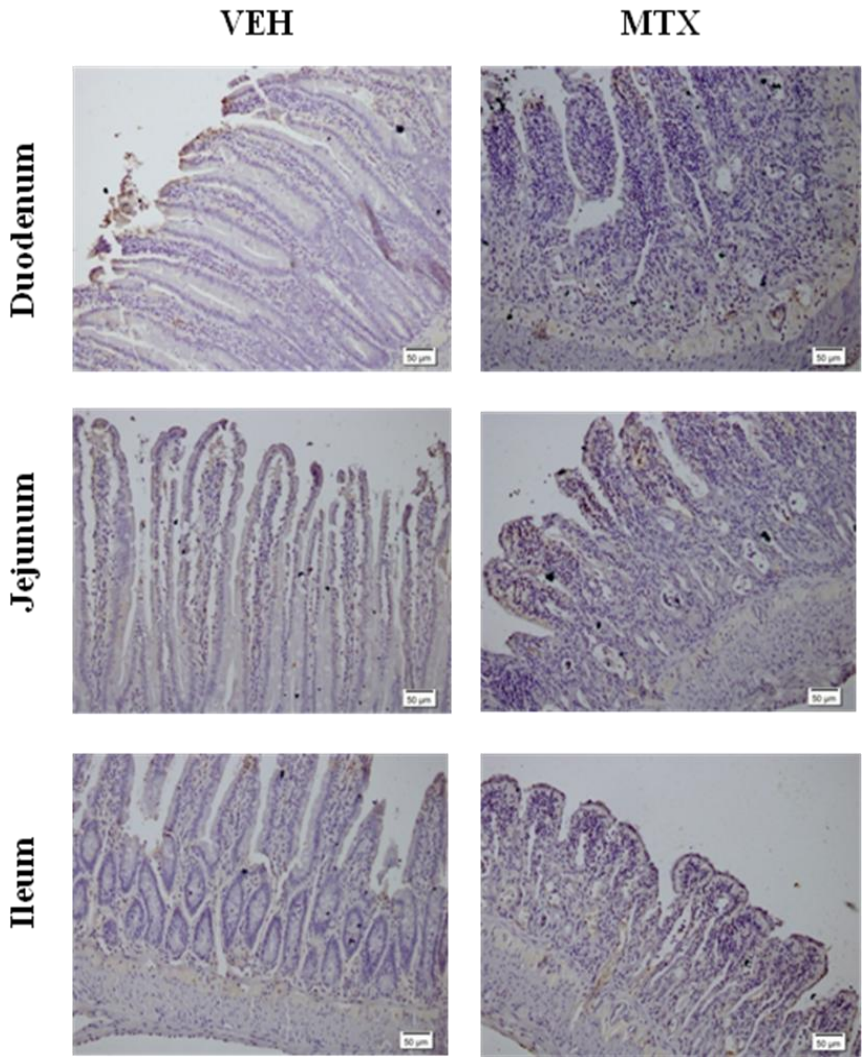
MTX treatment resulted in the activation of caspases 9 and 3

Immunohistochemistry of for active caspases 9 and -3 in the small intestines of control rats revealed basal caspase 9 (Figure: 6.4.3) and caspase 3 (Figure: 6.4.5) expression. This finding suggests that in the small intestinal epithelial cells partial activation of caspase-3 takes place normally. Caspase-3 may have important roles in physiological progression of IEC apoptosis and replacement of IEC .In the MTX treated rat intestines, the staining for caspases was more intense in the villi enterocytes, suggesting that MTX causes activation of caspases 9 and 3, thereby promoting apoptosis.

The activation of the upstream caspase, (caspase-9) and the executor caspase, caspase 3 were analysed in the cytosolic fractions of the small intestines by western blot. Cleaved (activated) caspase 9 and 3 were negligible in the control rats. However, in the MTX treated rat intestines the protein levels of active caspase 9 (Figure: 6.4.4) was increased by 2.5 fold (2.8 ± 0.01 vs. 0.0006 ± 0.0002) and caspase 3 (Figure: 6.4.6) by 4 fold (4.2 ± 0.01 vs. 0.0006 ± 0.0002) as determined by densitometry of caspase 9 and caspase 3 specific bands using western immunoblot.

Gene expression of caspase 3 (CASP3) was 3.33 fold (0.003 ± 0.0009 vs. 0.011 ± 0.003, $P = 0.004$) decreased (Figure: 6.4.7) and the total caspase-3 activity was significantly decreased by 3 fold (0.029 ± 0.01 vs. 0.062 ± 0.02, $P = 0.04$) (Figure: 6.4.8) in MTX treated samples as compared to control. This may be an adaptive mechanism in order to check extensive apoptosis initiated by MTX.

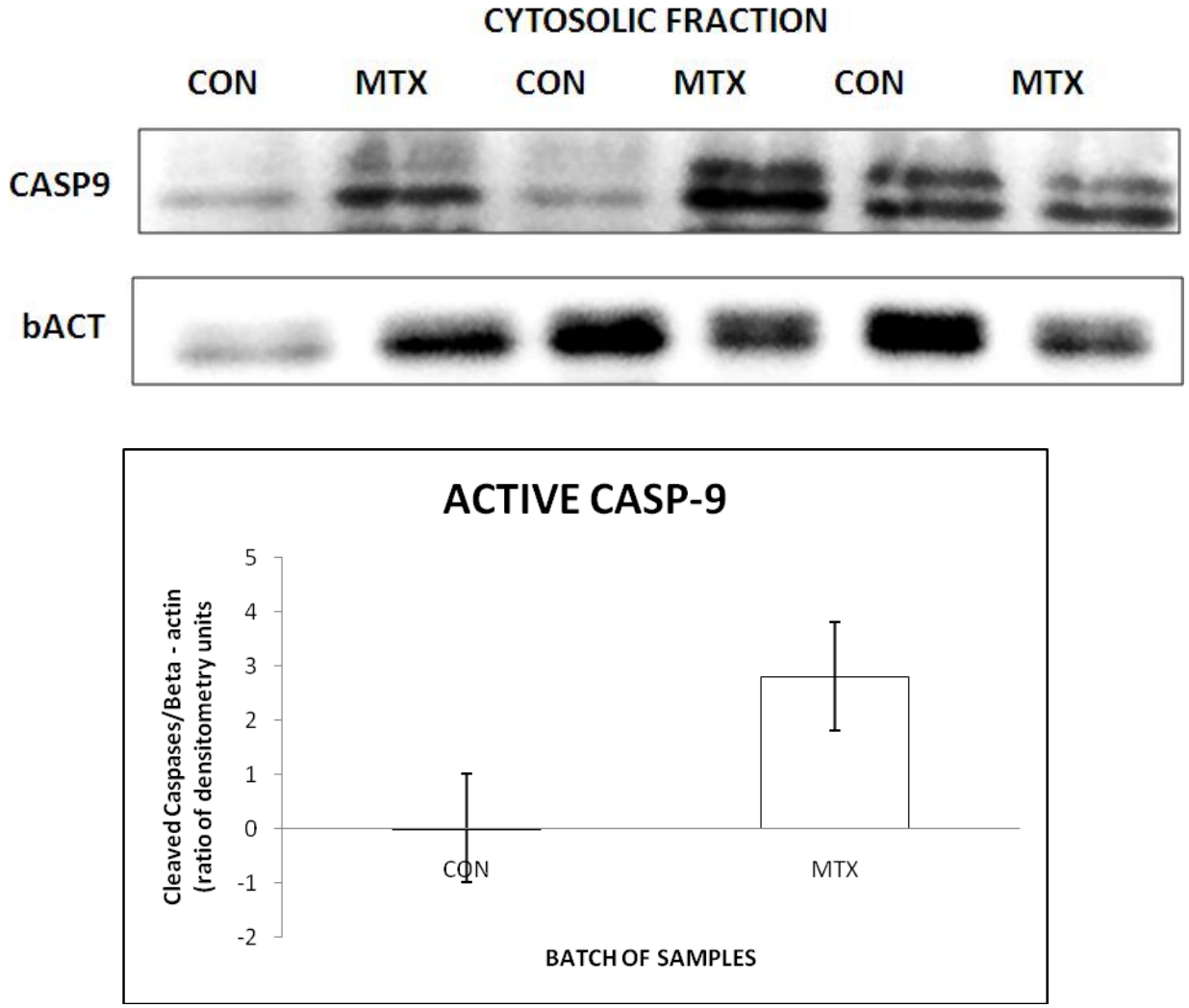
Figure: 6.4.3 **CASP-9 IHC**



Representative image of caspase 9 (CASP9) protein expression in the small intestine segments of rats 24 hours after the treatment with vehicle and MTX by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of CASP9 when compared to basal immunostaining in control rat small intestine tissue treated with vehicle.

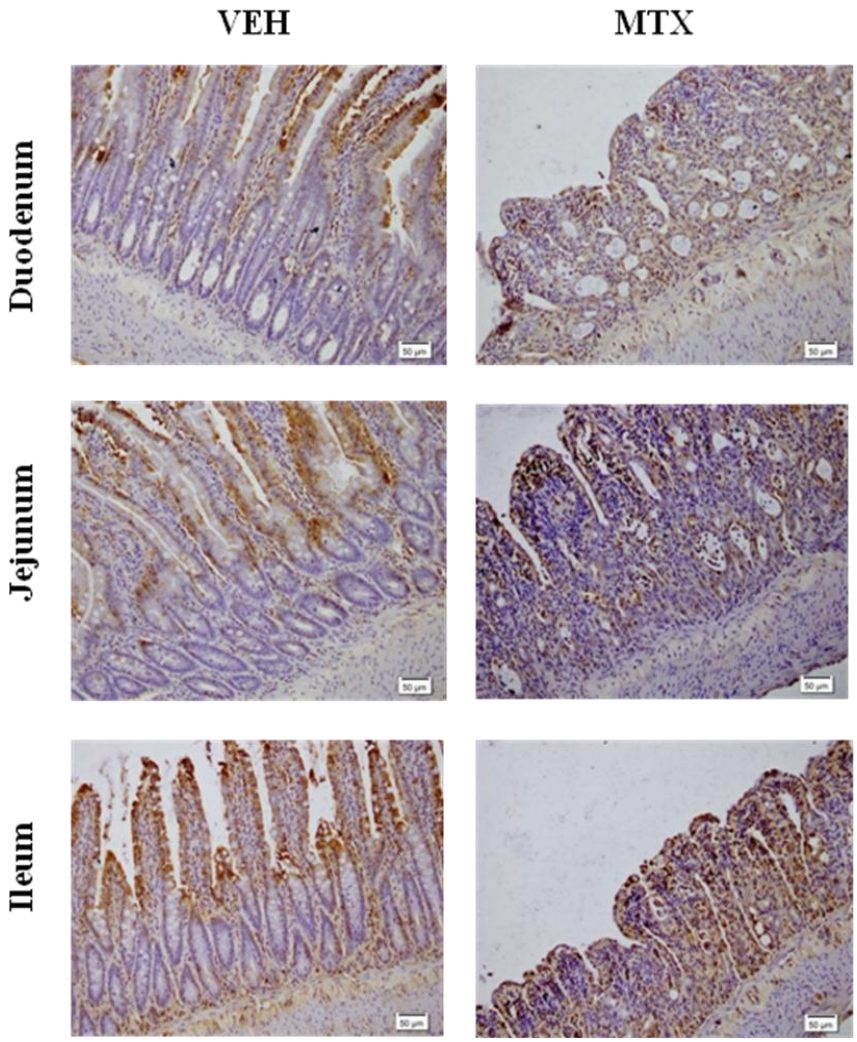
Site	Duodenum	Jejunum	Illeum
VEH	+/-	+/-	+/-
MTX	+++	++	+++

Figure: 6.4.4



Representative blot and corresponding graph of active CASP-9 protein in cytosol fraction of control and MTX treated small intestine mucosa tissue samples after 24hours of final dose. There is increased active CASP-9 in cytosol fraction of MTX than control. Data represent mean ± SD, N = 3 in each group.

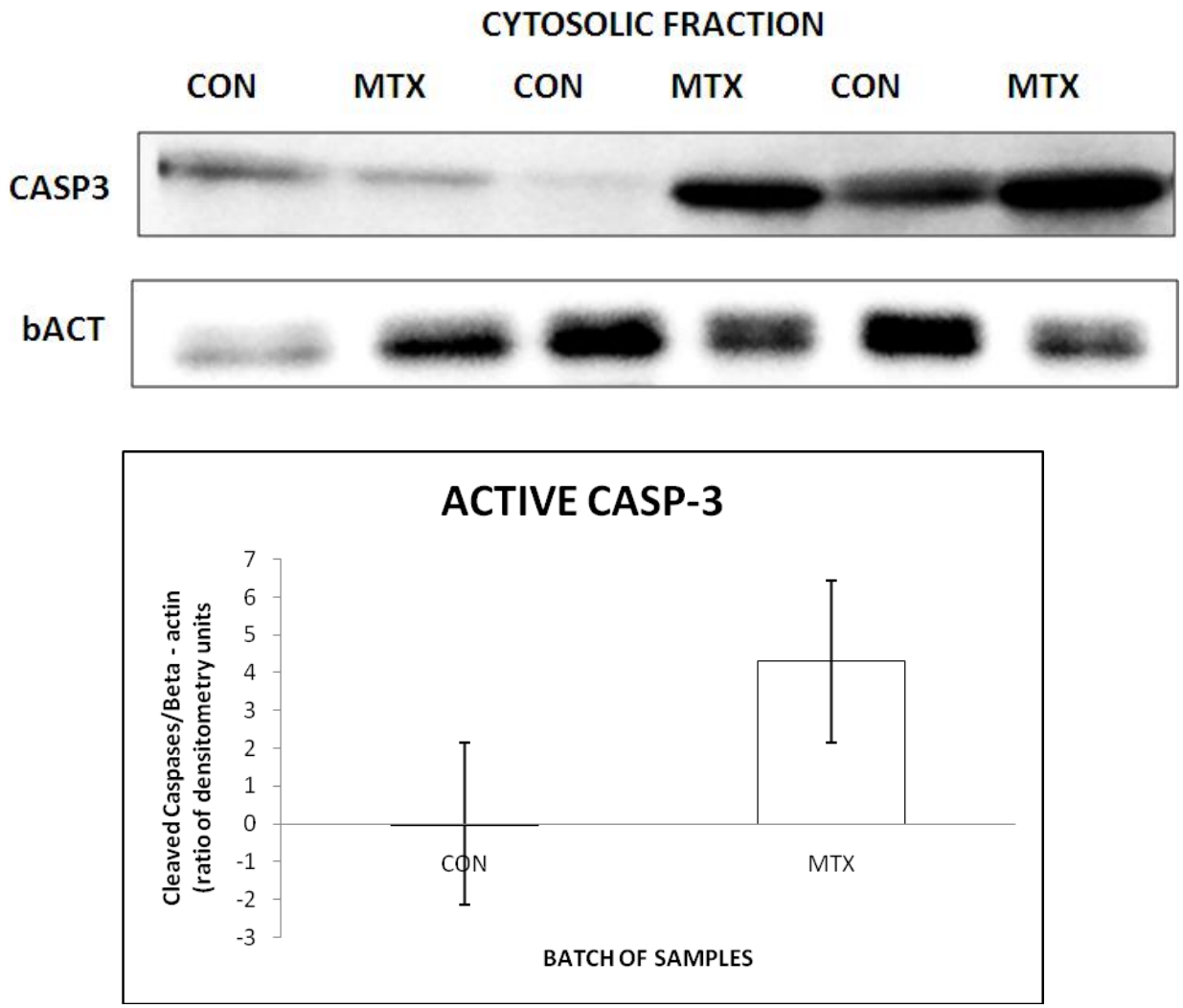
Figure: 6.4.5 **CASP-3 IHC**



Representative image of caspase 3 (CASP3) protein expression in the small intestine segments of rats 24 hours after the treatment with vehicle and MTX by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of CASP3 when compared to basal immunostaining in control rat small intestine tissue treated with vehicle.

Site	Duodenum	Jejunum	Illeum
VEH	+/-	+	+
MTX	++	+++	+++

Figure: 6.4.6



Representative blot and corresponding graph of active CASP-3 protein expression in cytosol fraction of control and MTX treated small intestine mucosa tissue samples after 24hours of final dose. There is increased active CASP-3 in cytosol fraction of MTX than control. Data represent mean ± SD, N = 3 in each group.

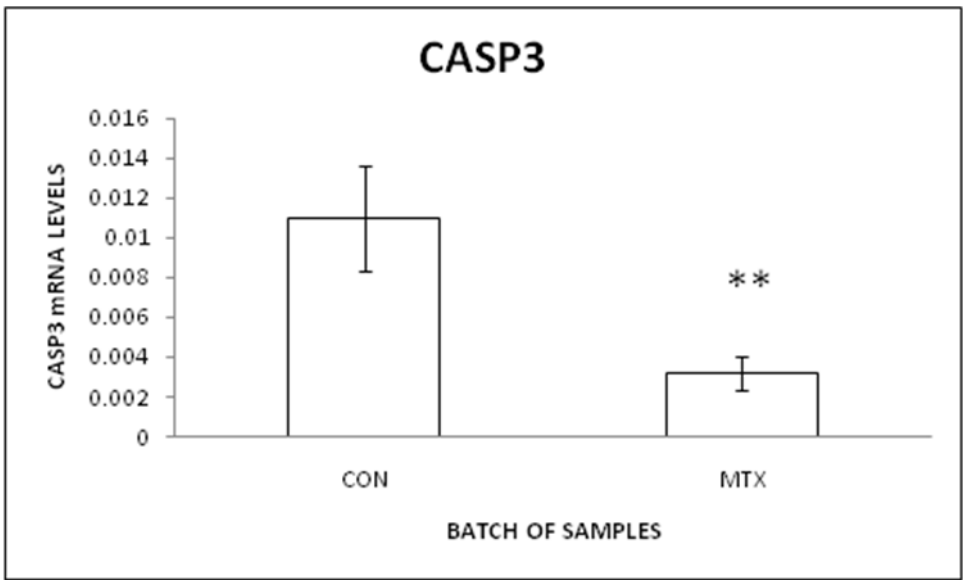


Figure: 6.4.7 - Effect of MTX on CASP3 mRNA levels. RT-PCR analysis of CASP3 mRNA expression after 24hours of final dose of MTX and vehicle treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

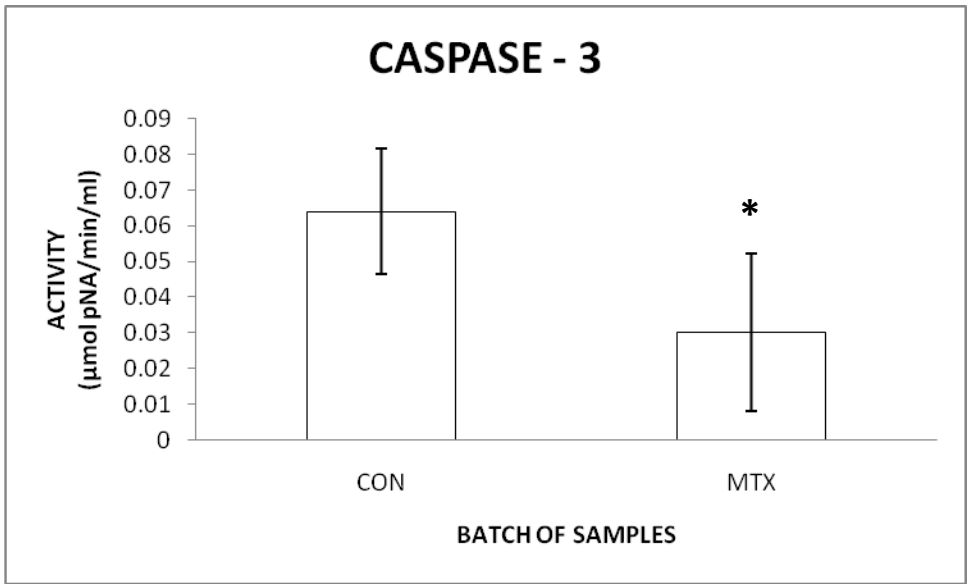


Figure: 6.4.8 - Effect of MTX on caspase-3 (CASP-3) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

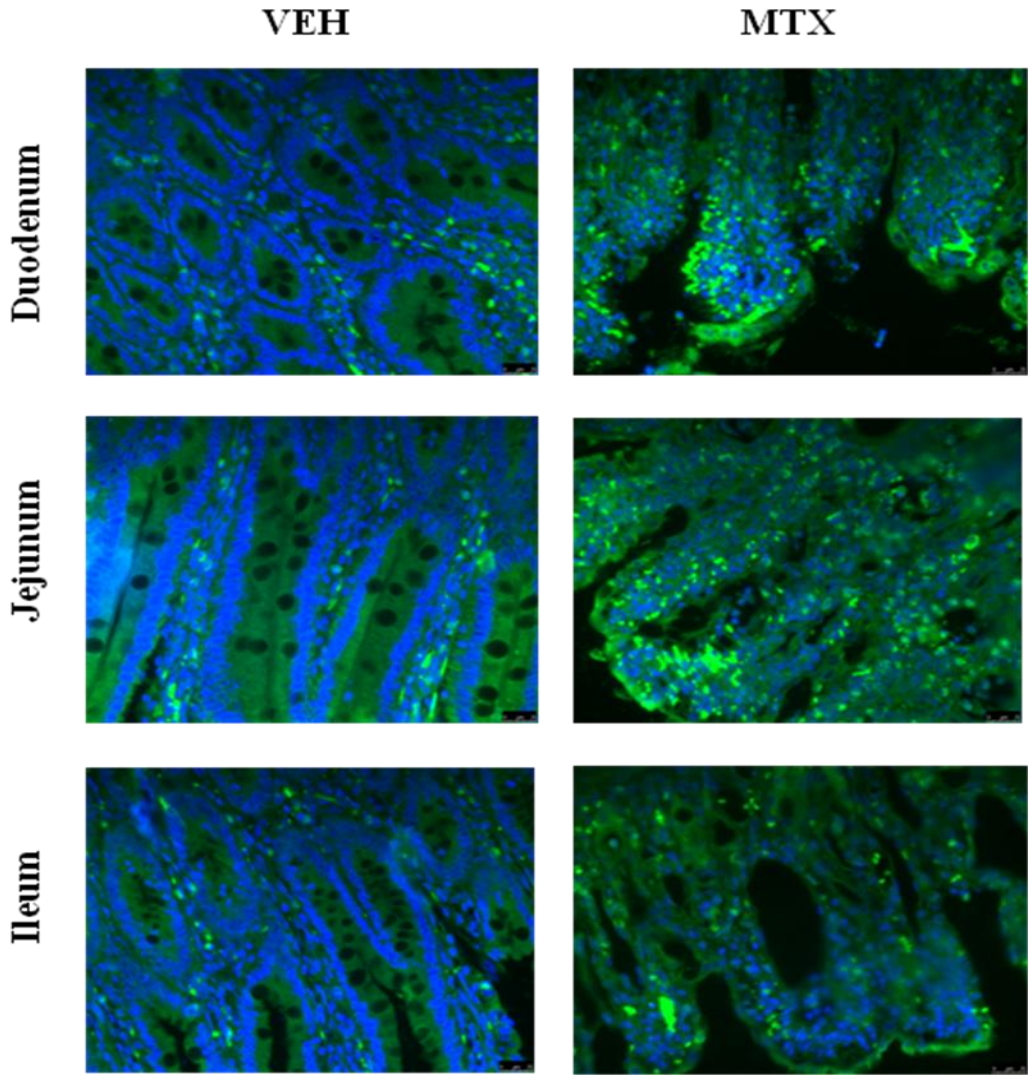
MTX treatment resulted in extensive PARP cleavage

Caspases are well-known proteases capable of PARP cleavage and the promotion of apoptosis. Therefore, cleavage of PARP-1 by caspases is considered to be a hallmark of apoptosis. The cleavage of PARP-1 by these caspase 3 results in the formation of 2 specific fragments: an 89-kD catalytic fragment and a 24-kD DBD. PARP-1 has been implicated in the process of apoptotic cell death following cellular injuries inflicted by the inflammatory response, ROS, and ischemia reperfusion. Therefore, we next assessed PARP expression by immunofluorescence, immunohistochemistry and western blot. Immunofluorescence staining for PARP in the intestinal tissues revealed basal PARP expression in the enterocyte nucleus of control rat intestines and was increased after MTX treatment (Figure: 6.4.9).

PARP was detected in the small intestines by immunohistochemistry (Figure: 6.4.10). PARP staining was detected in the gut of control rats and was very intense in the MTX treated rat. PARP was detected mainly at the damaged tip of the villi, and localized in the necrotic epithelial cells. Furthermore, nuclei of intestinal epithelial cells and stromal cells also displayed strong immunostaining for PARP in the MTX treated rats.

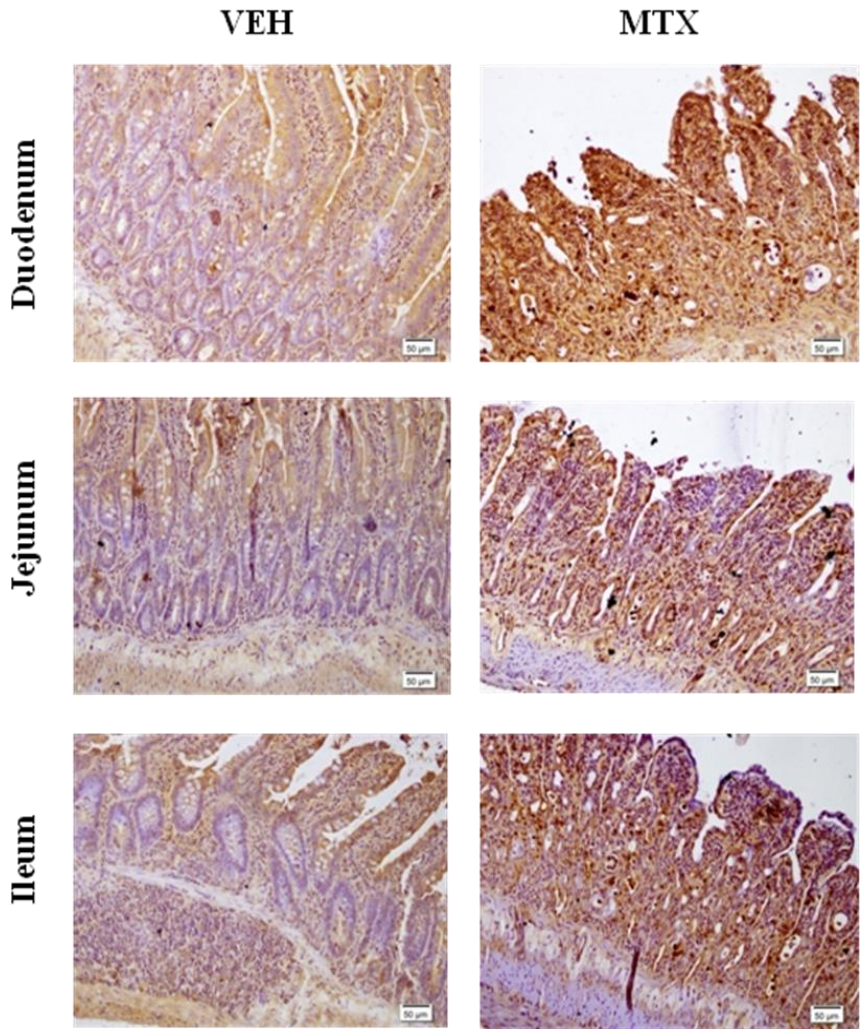
Next, we assessed PARP cleavage by Western blot. Western blots of PARP protein in nuclear fractions of intestines of control rats showed the presence of full length PARP 116-kDa, and cleaved PARP was almost not detectable. Whereas, in the nuclear fraction of small intestines of MTX treated rats full length PARP almost disappeared and there was significant appearance of the 85-kDa (breakdown fragment (Figure: 6.4.11). These finding suggest a role for caspase induced PARP cleavage in MTX induced small intestinal damage.

Figure: 6.4.9 PARP activation in nucleus by IF



Representative image of Poly ADP-ribose polymerase (PARP) expression on nucleus in the small intestine segments of rats 24 hours after the treatment with vehicle and MTX by immunofluorescence analysis (40X). MTX treated rats shown increased immunofluorescence of PARP expression (green) on nucleus (blue) when compared to basal immunostaining in control rat small intestine tissue treated with vehicle.

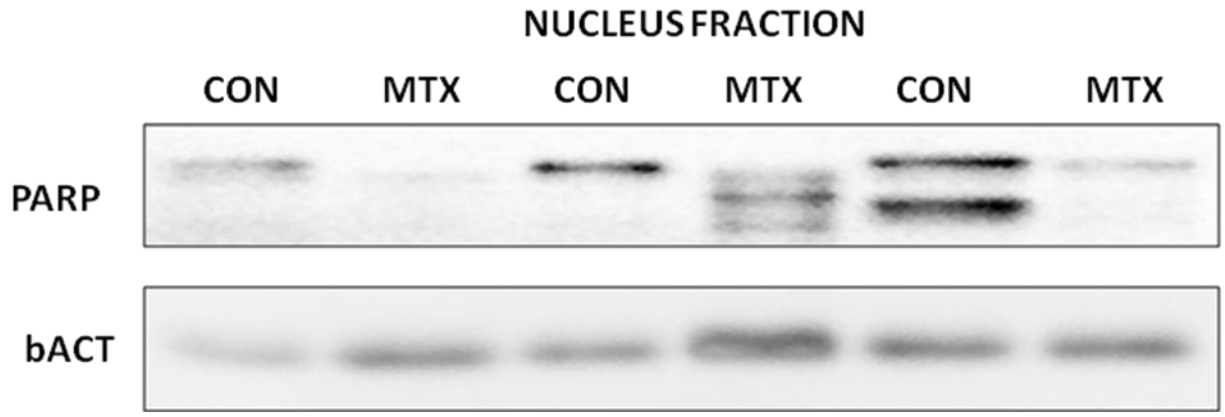
Figure: 6.4.10 **PARP IHC**

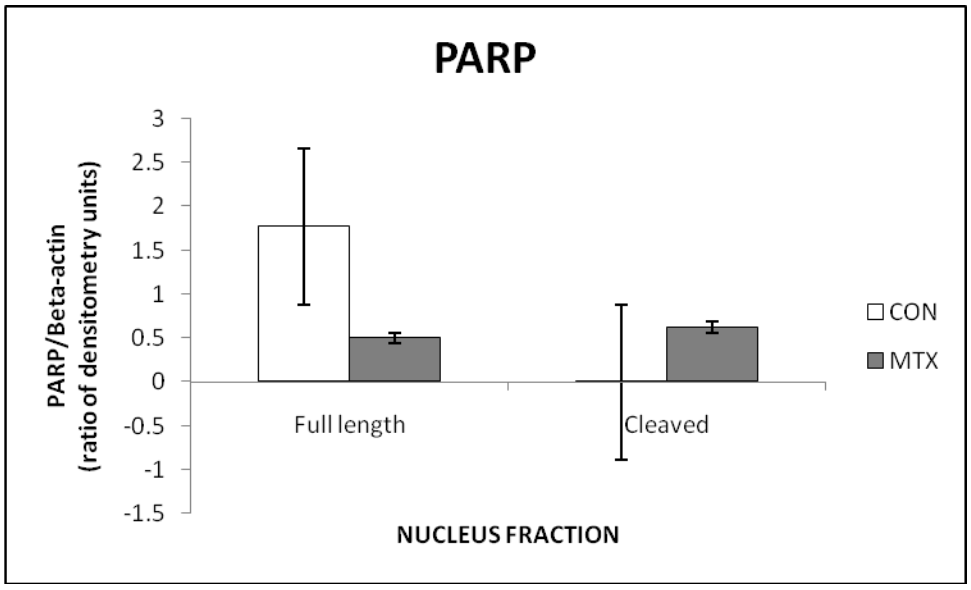


Representative image of Poly ADP-ribose polymerase (PARP) protein expression in the small intestine segments of rats 24 hours after the treatment with vehicle and MTX by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of PARP when compared to basal immunostaining in control rat small intestine tissue treated with vehicle.

Site	Duodenum	Jejunum	Illeum
VEH	+/-	+/-	+/-
MTX	++	+++	+++

Figure: 6.4.11





Representative blot and corresponding graph of active PARP protein in nucleus fraction of control and MTX treated small intestine mucosa tissue samples after 24hours of final dose. There is decreased full length PARP and increased cleaved active PARP in MTX than control. Data represent mean ± SD, N = 3 in each group.

MTX treatment resulted in decreased DNA content and increased DNA fragmentation

DNA was isolated from the mucosal samples and the concentration was measured using nanodrop with the factor 50 as microgram/ microliters. We found a decrease in the concentration of the DNA in MTX (2.71 ± 2.02 vs. 8.59 ± 1.46, P = 0.02) as compared to control. (Figure: 6.4.12a)

Nuclear DNA degradation is a critical event in the mechanism of cell death. DNA damage can be seen as DNA laddering. We analysed the effect of MTX on DNA fragmentation. There was intact DNA without any laddering in the control group. There was substantial increase in internucleosomal DNA fragmentation evident from DNA laddering pattern in the agarose gel induced by MTX treatment. (Figure: 6.4.12b)

Figure: 6.4.12a - DNA content in the intestinal mucosa of MTX treated rat and control rat

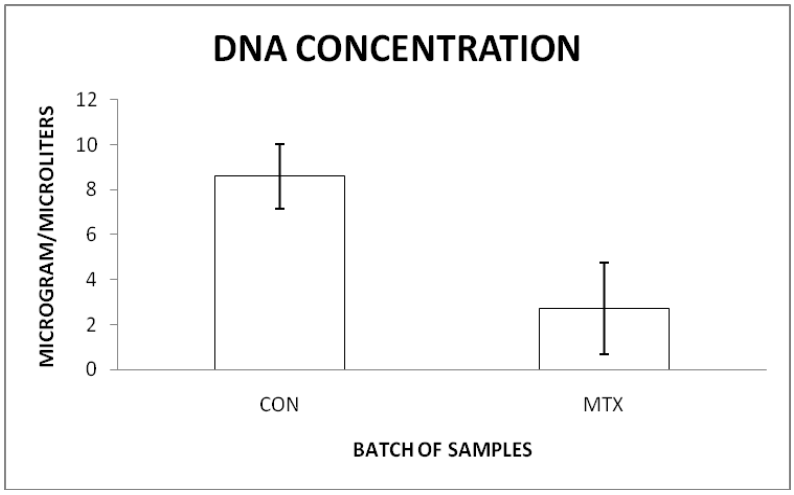
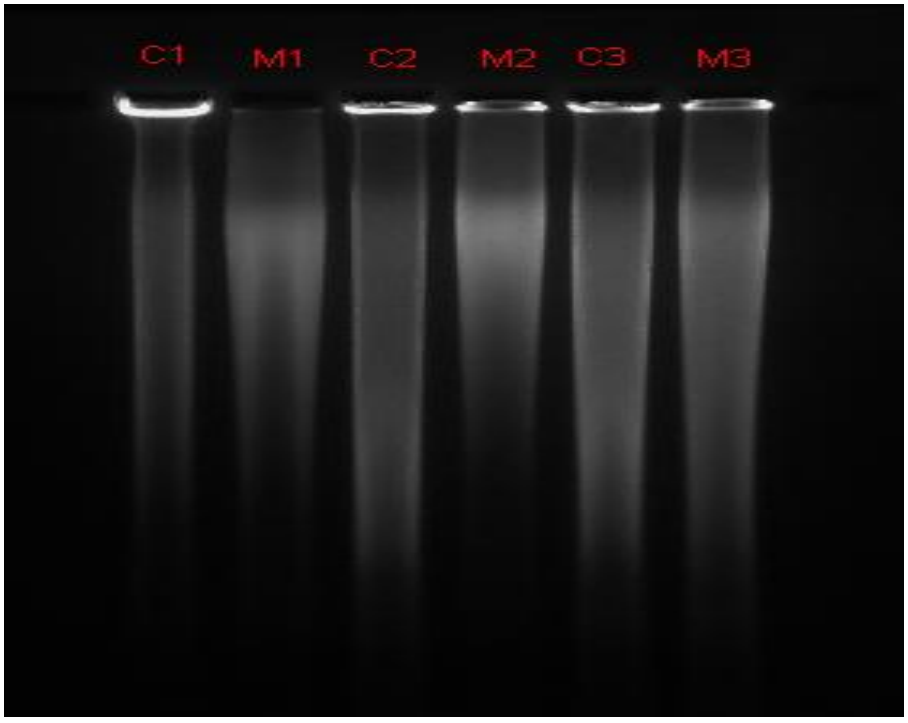


Figure: 6.4.12b DNA FRAGMENTATION ASSAY



Effect of MTX on DNA fragmentation and respective concentration. C1, C2, C3 – control samples. M1, M2, M3 – methotrexate treated samples. DNA was fragmented in MTX treated samples and DNA content was decreased significantly when compared to control.

6.4.6 DISCUSSION

The intestine is unique among solid organs because of its high cell turnover, with epithelial proliferation, differentiation and cell elimination occurring within a few days (407). Although cells can be removed from the intestinal crypts and villi by either apoptosis or exfoliation into the lumen, mathematical models suggest that apoptosis accounts for the bulk of cell loss in the intestine (408). Both increased and decreased gut epithelial apoptosis have been implicated in the pathophysiology of multiple local and systemic disease states including (but not limited to) sepsis, cancer, radiation-injury, inflammatory bowel disease, ischemia/reperfusion, burn injury, and necrotizing enterocolitis (409). Depending on the inciting event, gut epithelial apoptosis can occur via the receptor mediated (extrinsic) pathway, the mitochondrial (intrinsic) pathway or a combination of both pathways (410).

NO is a key mediator of intestinal cell and barrier function (102, 218, 247). When NO is present in excess, however, the result is barrier dysfunction (218). NO production acts as a promoter of different species that may lead to deleterious responses such as necrosis or apoptosis (411). Moreover, the importance of NO- and peroxynitrite mediated apoptosis has been recognized clinically in a variety of dysfunctions and transplant diseases. Many apoptotic factors activate programmed cell death via cytochrome c release, among them sustained mitochondrial NO generation and peroxynitrite formation. Peroxynitrite is a highly reactive molecule that exerts its cytotoxic effects by inducing mitochondrial membrane depolarizationand by cytochrome c release (412).

NO can induce apoptosis directly or indirectly through its conversion to peroxynitrite. NO can reversibly inhibit enzymes that produce free radical intermediates, such as those involved in the mitochondrial electron transport chain (ETC). When NO inhibits cytochrome-c oxidase, superoxide may transiently leak from the ETC, leading to the formation of peroxynitrite (292). While the reactions of NO with enzymes are usually reversible, the reactions of peroxynitrite are not. Through oxidation of critical cysteine residues, peroxynitrite irreversibly inactivates many components of the ETC, including complex I, complex II, complex III and complex V (291), leading to inhibition of oxidative phosphorylation and ultimately, cell death (182, 411). Interestingly, in our study we found drastic decrease in the activities of the ETC complexes in the small intestine of rats after treatment with MTX. This was accompanied by PON overproduction as evidenced by 3NT, suggesting that the loss of activities of these enzymes may be due to their nitration. In an earlier study we have demonstrated loss of mitochondrial function as evidenced by reduced RC, MTT reduction and mitochondrial swelling (23). These findings suggest that PON overproduction, disruption of etc complexes and loss of mitochondrial function may play a role in MTX induced enterocyte apoptosis and small intestinal injury.

Intestinal basal stem cell is susceptible to the toxicity of chemotherapeutic drugs for its character of rapid turning-over, leading to cell apoptosis in gut tissue and resulting in mucositis. Two key events in the mitochondrial pathway of apoptosis include cytochrome *c* release from mitochondria and activation of caspase-3 (188, 190). Therefore we assessed the effect of MTX on cytc release and caspase activation. We analysed cyt c protein expression by IHC and western blot analysis of cyt c protein in cytosolic fraction and mitochondrial fractions in the small intestines. In the control intestines, cyt c was almost exclusively found in the mitochondrial

fraction, with very minimal amounts in the cytosolic fraction. On the other hand, in the MTX treated rat intestines, cytc was predominantly seen in the cytosolic fraction and small amounts in the mito suggesting cyt c release from mito into the cytosol.

Cytochrome *c* (cyt *c*) is a small globular heme protein present at high concentrations (~ 1 mM) in the intermembrane space of mitochondria (413). The primary role of cyt *c* is to shuttle electrons between respiratory complex III (cytochrome bc_1 complex) and complex IV (cytochrome *c* oxidase), where oxygen is reduced to water (413). However, possible alternative functions of cyt c have been recognized recently. As cyt *c* is abundant in the mitochondrial intermembrane space, it is a potential target of the diffusible PN generated within or near the mitochondria. $ONOO^-$ formation induces cytochrome *c* release, an indicator of mitochondrial distress and potential inducer of cell death (414).

Cyt *c* nitration by PN *in vitro* has been previously studied by Radi et al (200). Respiratory cytochrome *c* (C*c*) is the main target for RNOS in mitochondria, where it is both nitrated and nitrosylated *in vivo* (115, 415). The preferential nitration of cytochrome by peroxynitrite-derived radicals in tyrosine residues (Tyr-74) leads to a conformational change that causes the displacement of the sixth ligand to the heme (Met-80) and a gain in peroxidase activity, the conformational change induced by nitration in cells also facilitates its translocation to the cytosol (even in non-apoptotic cells) (416).

It is suggested that the decrease of mitochondrial cytochrome *c* even in the range of 10–20% s may have a significant effect on initiation of caspase activation and apoptosis within

cardiomyocytes. Early changes in approximately 50 kDa and approximately 25 kDa protein were observed in tyrosine nitration along with a loss of cytochrome c (417). In the present study as cyt-c release is accompanied by overproduction of PON (as evidenced by increased 3NTin the small intestines of MTX treated rats), we speculate that PON induced nitration of Cytc may be responsible for its translocation into the cytosol, thereby initiating apoptosis.

Caspases are well-known proteases the promotion of apoptosis. After apoptosis is triggered, caspases are activated by the cleavage of proenzymes (zymogens) into distinct subunits that rearrange to form the active cysteine proteases (418). Caspases transduce and augment the apoptotic signal by activation of other caspases. Cytosolic cyt c acts in concert with apoptosis activating factor-1 (Apaf-1) to activate procaspase9. Active caspase 9 is an initiator caspase, which in turn activates effector caspases, such as caspases 3 and 7 Caspase3 is a member of caspase family and plays a central role in the execution-phase of cell apoptosis and cleaves the majority of cellular substrates in apoptotic cells (419). Therefore we analysed the expressions of caspases 3 and 9 by WB, IHC. In the normal rat intestines here was basal expression of the caspases suggesting that they may have physiological roles. In the small intestine, cells generated from stem cells at the base of the crypt differentiate into absorptive cells and are finally lost from the tips of villi by apoptosis, resulting in replacement of lining cells every two to three days. Although cells can be removed from the intestinal crypts and villi by either apoptosis or exfoliation into the lumen, mathematical models suggest that apoptosis accounts for the bulk of cell loss in the intestine (420). Therefore it is not surprising to find caspase positive (apoptotic cells) at the tips of villi as well as in crypts in normal rat intestines. MTX treatment resulted in increased expression and activation of caspases 9 and 3 in the small intestines suggesting

enhanced apoptosis may be one of the mechanisms by which MTX exhibits GI toxicity. Several studies showed that caspase-3 activity was increased in the process of intestinal apoptosis during chemotherapy, including 5-FU treatment (406). We observed a marked increase in immunopositive cells for cleaved caspase-3, the active form of caspase-3, which localized to the intestinal crypt in a manner consistent with the localization of apoptotic cell. MTX treatment significantly lowered the mRNA levels of caspase3. The relevance of this finding is not clear. However, we suggest that this may be defense mechanism to prevent excessive apoptosis induced by MTX.

Caspase mediated apoptotic cell death is accomplished through the cleavage of several key proteins required for cellular functioning and survival. PARP-1 is one of several known cellular substrates of caspases, mainly 3 and 9. Activated caspases may trigger the cleavage of PARP (421), and may also act upon a variety of intracellular targets to promote apoptosis (422).

The PARP family consists of 17 members which have different structures and diverse functions in cells. PARP-1 is an abundant nuclear enzyme with approximately1-2 million copies in the cell which account for ~85% of total cellular PARP activity (400). PARP-has a wide range of physiological as well as pathological functions. PARP-1 facilitates DNA repair and has been implicated in the process of apoptotic cell death following cellular injuries inflicted by the inflammatory response, ROS, and ischemia reperfusion. Under basal conditions, the primary function of PARP-1 is to detect and repair DNA damage. However, cells with severely damaged DNA have amplified PARP-1 activity resulting in high NAD+ consumption (depleting ATP pools). If unchecked, this activity inevitably leads to passive necrotic cell death (resulting from

prolonged ATP depletion) (423). This process is blocked by rapid cleavage and inactivation of PARP-1 by the action of caspases. However, insults which initiate necrosis cause PARP-1 overactivation that proceeds unchecked due to inadequate caspase activation, lower PARP-1 cleavage and less PARP-1 24-kD fragment formation (400).

Having shown caspase 9 and 3 activation in the MTX treated rat intestines, we next assessed the expression of full length PARP-1 by IF, IHC and cleaved PARP-1 by WB. IF and IHC showed overexpression and nuclear translocation of PARP-1 in the MTX treated rat intestines. Western blot analysis of nuclear extracts from control rat intestines presented only 1 band at 113 kDa corresponding to the full length of PARP-1, while cleaved PARP was almost absent. Western blots of PARP protein in nuclei isolated from small intestines indicated that there was no disappearance of the 116-kDa active form of PARP and no significant appearance of the 85-kDa breakdown fragment in the control rats. After MTX treatment, significant cleavage of PARP-1 was seen as shown by the formation of 2 specific fragments: an 89-kD catalytic fragment and a 24-kD DBD, in addition to moderate amounts of full length PARP. It has been shown that cleavage of PARP by caspases to generate 89-kDa and 24-kDa fragments, is a hallmark of apoptosis and that this cleavage is thought to be a regulatory event for cellular death.

Although cleavage of PARP-1 by caspases is a universal phenomenon during apoptosis, the significance of this cleavage in vivo is largely unknown. It is postulated that PARP-1 cleavage might occur in cells undergoing apoptosis to inactivate their capacity to repair DNA in order to preserve energy pools (424). An 89-kD catalytic fragment and a 24-kD DBD are 2 specific fragments obtained as a result of PARP-1 cleavage by caspase 3(400). The 89-kD fragment

containing AMD and the catalytic domain of the enzyme has a greatly reduced DNA binding capacity and is liberated from the nucleus into the cytosol. The 24-kD cleaved fragment with 2 zinc-finger motifs is retained in the nucleus, irreversibly binding to nicked DNA where it acts as a transdominant inhibitor of active PARP-1. Importantly, irreversible binding of the 24-kD PARP-1 fragment to DNA strand breaks inhibits DNA repair enzymes (including PARP-1) and attenuates DNA repair (also conserving cellular ATP pools) (425). Thus, Cleavage and inactivation maintains the cellular ATP stores indispensable for apoptosis to proceed properly. Consistent with these hypotheses, cell lines expressing a caspase-resistant PARP-1 by mutating the DEVD214 site displayed increased apoptosis and necrosis after TNF-a treatment (424). These studies indicate that the caspase cleavage of PARP-1 is an important regulatory event in cellular functions.

It must be stressed that PARP activation and PARP cleavage are fundamentally distinct events, linked to completely different modes of cell demise, that is, either necrotic or apoptotic. The relationship between PARP activation and PARP cleavage, apoptosis vs. necrosis, is complex, but accumulating evidence suggests that PARP activation contributes to cell necrosis, while PARP cleavage (leading to a decreased PARP-1 activity) may serve as a protective mechanism (to prevent necrosis by cellular energy exhaustion) and thereby permit apoptosis. Studies have shown that PARP cleavage functions as a molecular switch between apoptotic and necrotic modes of cell death (426).

In the present study we have demonstrated increased PARP cleavage in the small intestines of MTX treated rats by different methods. Our studies demonstrate that IEC constitutively contain

the machinery of cell death, which is activated by MTX, leading to caspase activation.It is therefore proposed that caspase 3 activation and PARP cleavage and apoptosis contribute to MTX induced small intestinal injury.

In addition to apoptotic cell death, PARP-1 cleavage seems to regulate expression of inflammation mediators through its role in coactivation with NF-kB (427). As a co-activator of nuclear factor kappa B (NF-κB), PARP-1 may potentiate injury by promoting the synthesis of pro-inflammatory mediators at the site (424). As PARP-1 fragments can costimulate NF-kB activity, it is reasonable to speculate that cleaved fragments of PARP-1 might modulate the interaction of p300 and NF-kB with the basal transcription machinery (428-429). However, the exact molecular mechanisms have yet to be investigated. Studies have shown that NFkB transcriptional activity was impaired in *PARP-1*KI/KI cells in response to inflammatory stress (424, 430). These results suggest that the PARP-1 cleavage event is physiologically relevant in the regulation of an inflammatory response in vivo.

Indeed, we found that enhanced PARP cleavage is accompanied by increased NF-kB transcriptional activity in the small intestines of MTX treated rats, thereby suggesting a role for PARP-1 in inflammation. In certain inflammatory conditions such as coeliac disease, nematode infections, and graft versus host disease, numbers of apoptotic nuclei were reported to be increased in villus epithelial cells, indicating that apoptosis has important roles not only in physiological replacement of villus epithelial cells but also in pathological conditions (421).
Nuclear DNA degradation is a critical event in the mechanism of cell death (431-432), and at least in part this process seems to be linked to mitochondrial dysfunction. There is good evidence

that exposure of cells to oxidant stress (e.g. H2O2, peroxynitrite, etc.) results in strand breaks in DNA. As MTX induced mucositis is associated with overproduction of ROS and RNS, we analysed DNA fragmentation by agarose gel electrophoresis of DNA isolated from rat intestine. In the control group, there was intact DNA without any laddering. MTX treatment resulted in substantial increase in internucleosomal DNA fragmentation evident from DNA laddering pattern indicating apoptotic death of enterocytes.

Apoptosis in the crypts and villus of the small intestine is reported to contribute to chemotherapeutic drug and radiation induced small intestinal injury (433). Augmentation of enterocyte apoptosis has been shown to one of the mechanisms by which chemotherapeutic drug induced mucositis (434). Inhibition of enterocyte apoptosis by different agents has been shown to improve intestinal recovery following chemotherapy -induced intestinal-mucositis in rats (58). Methotrexate, irinotecan, and 5-fluorouracil, cisplatin have been reported to mediate their GI toxicity by activation of the mitochondrial apoptotic pathway (23, 405-406, 435). This is evidenced by their induction of chromatin condensation, DNA fragmentation, caspase-3 activation and cytochrome c release. The key role of apoptotic pathway in chemotherapeutic drug uinduced mucositis is supported by the observations that combined treatment of the CT with antioxidants such as NAC, carnitine, genestein, vitamin C and tocopherol attenuated the apoptotic pathway and prevented SI injury (433, 436-439). The findings of our study is supported by earlier reports that IEC-6 cells exposed to MTX, the chromatin condensation, DNA fragmentation, caspase-3 activation and cytochrome c release were observed (440). The MTX-induced apoptosis of IEC-6 cells was shown to be depressed by AGE (440-441).

In summary, apoptosis appears to play a role in the occurrence of intestinal mucositis induced by MTX chemotherapy. The mitochondrial pathway of apoptosis appears to play a predominant role in MTX mucositis as evidenced by cytochrome c release, activation of caspases 9 and 3, PARP cleavage, and DNA fragmentation.

STUDY – V

Protective effect of Aminoguanidine, selective inhibitor of iNOS on MTX induced mucositis

STUDY 5: Effect of pretreatment with aminoguanidine, a selective inhibitor of iNOS on MTX induced nitrosative stress, upregulation of NFkB inflammatory pathway and apoptotic pathway, and small intestinal mucositis

6.5.1. ABSTRACT

Aminoguanidine, a selective iNOS inhibitor, has powerful antioxidant property in addition to its potency to attenuate nitrosative stress. Our earlier studies have shown that MTX induced small intestinal damage is due to increased oxidative stress and nitrosative stress. In the present study we analyzed the protective effect of aminoguanidine on MTX treated enteritis. To study this, rats were pretreated with aminoguanidine 30mg or 50mg /kg body weight 1 hour before the treatment of MTX 7 mg/kg body weight. Control rats receive vehicle alone. A group of rats also received aminoguanidine 30mg or 50mg/ kg body weight. After 24 hours of the final dose of MTX the rats were sacrificed and the small intestine tissue was removed. The segments of small intestine were stored for histology and histochemistry studies. The mucosa was collected and used for rest of the experiments. The small intestine of rats in MTX treated group showed obvious mucosa atrophy and villi loss with the degeneration and necrosis of epithelium cells and infiltration of inflammatory cells. When the animals were pretreated with aminoguanidine, the development of these intestinal lesions was prevented in a dose-dependent manner. As expected, AG pretreatment significantly decreased MTX induced iNOS upregulation, NO overproduction, and 3NT. AG pretreatment attenuated MTX induced loss of activities of antioxidant enzymes including catalase, GST, CA, as well as ETC complexes IV and V and TCA enzymes, aconitase, and SDH. AG pretreatment attenuated MTX induced upregulation of NFκB and its proinflammatory target genes protein expressions, iNOS, TNF, COX-2 and PLA_2. AG

pretreatment attenuated MTX induced mitochondrial apoptotic pathway, CYT-C, CASP-9, CASP-3, PARP cleavage, and DNA fragmentation. However, AG pretreatment had no significant effect on the mRNA expressions of these proteins. In conclusion, AG pretreatment improved MTX induced morphological changes in the small intestine inhibited the NFkB inflammatory pathway and apoptotic pathway. Thus, aminoguanidine has a protective role in MTX induced mucositis.

6.5.2. INTRODUCTION

Chemotherapy is one of the most effective treatments for patients with cancer. However, the cytotoxicity of chemotherapeutic agents to normal tissues is a critical factor that undermines the curative potential of chemotherapy. Chemotherapy-induced alimentary mucositis is an extremely common condition that is caused by a breakdown of the mucosal barrier. It occurs in between 40 - 100% of cancer patients depending on the treatment regimen. Symptoms typically include pain from oral ulceration, vomiting and diarrhea (442). Alimentary mucositis often necessitates chemotherapy reductions or treatment breaks, overall potentially compromising survival outcomes. Consequently, alimentary mucositis creates a burden not only on patients' quality of life but also on healthcare costs. Despite this, currently, there is no clinically effective localised/pharmacological therapy intervention strategy to prevent alimentary mucositis.

To achieve the method for selective killing of cancer cells without causing side effects of chemotherapeutic agents, it is important to elucidate the mechanism of cell death induced by the agents. Studies have shown that NO plays a role in intestinal mucositis (102, 253), the detailed mechanism by which NO contributes to SI injury is not fully understood. Mucositis has been

induced in the rats by the administration of chemotherapeutic agents, including methotrexate (225), Studies have shown that mucositis in this model mimics the symptoms and mucosal damage that occur in mucositis occurring in humans.

We used MTX in order to investigate in detail the role of NO in chemotherapy induced mucositis. In chapter 1 we have demonstrated increased protein tyrosine nitration, and selective nitration of proteins. We have also shown that NFkB and its target inflammatory cytokines TNF and COX-2 contribute to MTX induced mucositis (chap 3). In addition, we have demonstrated (chap 4) that apoptotic pathway plays a role in MTX induced SI injury as evidenced by the release of cytochrome c from the mitochondria to the cytosol, activation of caspases 9 and 3, PARP cleavage and DNA fragmentation.

In the gastrointestinal tract (GIT) NO shows a dual behavior: at physiological concentrations, released through the constitutive synthase (cNOS), it regulates house-keeping functions, whereas its overproduction by the inducible isoenzyme (iNOS) exhibits cytotoxic activity (103). Thus, non-selective NOS inhibitors such as L-NAME, L-NMMA, and other L arginine analogs not only inhibit the high output inducible NOS, but also inhibited constitutive NO synthesis that is critical to regulating tissue perfusion, microvascular permeability, and platelet and leukocyte-endothelial interactions (443). Thus, a specific inhibitor of iNOS will be needed, so that not compromising the activity of eNOS and the corresponding vasoregulatory function of •NO in the endothelium.

Aminoguanidine (AG) is a bifunctional molecule comprising the guanido group from L-arginine linked to hydrazine, and was first described by Corbett *et al.* (212) as a selective inhibitor of iNOS. Misko *et al.* (211) showed that AG selectively inhibits iNOS without increasing blood pressure; AG was 50-fold more effective on inhibiting the enzymatic activity of iNOS than eNOS or nNOS (212).

In this study, we tested whether the effects of AG on MTX induced SI injury. We used two doses of AG, 30 mg and 50 mg per kg body weight. The doses were chosen based on earlier studies (444-446).

6.5.3 AIM and OBJECTIVES

The present study was designed to evaluate the efficacy of aminoguanidine in the prevention of MTX mucositis.

The main objective of this study is to investigate the effect of AG pretreatment on

- Morphology of the small intestines
- Nitrated protein level in the small intestines
- Activities of enzymes that are reported targets of peroxynitrite
- NFkB inflammatory signaling pathway
- Apoptotic pathway

6.5.5. SUMMARY OF EXPERIMENT

Adult male wistar rats (200 – 250g) were treated as follows:

Group I : Control rats received vehicle alone

Group II: Rats received MTX 7mg/kg body weight intraperitoneally for 3 consecutive days.

Group III: Rats received aminoguanidine 30mg/kg body weight intraperitoneally for 3 consecutive days.

Group IV: Rats were pretreated with aminoguanidine 30mg/kg body weight 1 hour before the treatment of MTX 7mg/kg body weight intraperitoneally for three consecutive days.

Group V: Rats received aminoguanidine 50mg/kg body weight intraperitoneally for 3 consecutive days.

Group VI: Rats were pretreated with aminoguanidine 50mg/kg body weight 1 hour before the treatment of MTX 7mg/kg body weight intraperitoneally for three consecutive days.

After 24 hours of the final dose of MTX the rats were sacrificed and the entire length of small intestine tissue was removed, washed with saline and a piece of segments (duodenum, jejunum, ileum) was stored for histology, immunohistochemistry and double immunofluorescence studies. The mucosa scrapings was collected and used for the rest of the analysis.

Histology

The small intestine segments (duodenum, jejunum, ileum) was fixed in 10% buffered formaldehyde, dehydrated in graded ethanol and embedded in paraffin. Five-micron sections were cut on a microtome, mounted on clear glass slides and stained with haematoxylin and eosin. The sections were examined by light microscopy (Leica) and evaluated by pathologist in a blinded fashion.

Nitric oxide levels

Nitric oxide level was measured in mucosal homogenate by analysis of its metabolites (nitrate and nitrite levels) using copper cadmium alloy and griess reagent and the results were expressed as nmoles/mg protein.

Enzyme assays

The activities of ETC complex enzymes (IV, F1 ATPase), TCA cycle enzymes (aconitase, creatine kinase, succinate dehydrogenase, and glutamate dehydrogenase) and antioxidant enzymes (catalase, superoxide dismutase, glutathione s transferase, carbonic anhydrase) were measured by spectrophotometric method and expressed as respective units/mg protein.

Immunohistochemistry

Small intestine segments (duodenum, jejunum, ileum) was sectioned as 5microns and fixed on microscopic slides. Sections were incubated using iNOS, NTY, NFkB, COX2, CYTC, CASP3, CASP9, PARP primary antibody. The bound primary antibody was detected by the addition of secondary antibody conjugated with horseradish peroxidase polymer and DAB substrate. Later the slides were counterstained with Harris Haematoxylin and mounted with DPX mountant medium. Sections were examined under bright field light microscopy (Olympus - cellsens standard software) and evaluated by pathologist. The intensity of brown precipitate in the tissue sections indicates the presence of proteins.

IHC Scoring

Qualitative scoring was done based on the intensity of staining as score 1 for weak staining, score 2 for moderate staing and score 3 for strong staining. Quantitative scoring was done based on the positive cells as score 0 for <10% positive cells, score 1+ for 10-25% positive cells, score 2+ for 25-50% positive cells, score 3+ for 50-75% positive cells, score 4+ for >75% positive

cells. Quick score (Q) was calculate as results scored by multiplying the percentage of positive cells (P) by the intensity (I). Formula: Q = P x I; Maximum = 300 cells to be taken

Immunofluorescence

Small intestine segments (duodenum, jejunum, ileum) was sectioned as 5microns and fixed in microscopic slides. All the sections was incubated using NFkB and PARP primary antibodies and detected using respective fluorochrome conjugated secondary antibody. Nuclei counterstained by DAPI. Sections examined under fluorescence microscope. The intensity of red and green stain indicates the protein expression and the merge of red/green stain and blue stain indicated the location of protein on nuclei in small intestine sections.

PCR

RNA was isolated from small intestine mucosa samples using TRI reagent, cDNA was constructed and mRNA iNOS, NFkB, COX2, TNFA, CASP3 gene expression was analyzed using SYBR green reagent kit.

DNA fragmentation

DNA was isolated using TRI reagent. Isolated DNA was separated in 1% agarose gel that contained 0.05% ethidium bromide, using Tris-aceticacid-EDTA (TAE) buffer 40mM Tris base; Glacial acetic acid; 0.5M EDTA, Disodium Salt, Dihydrate; Final pH 8.0 . The separated bands were visualized and documented using an AlphaEase FC gel documentation system. The intact band indicates no fragmentation and the multiple bands seen in gel indicates fragmentation.

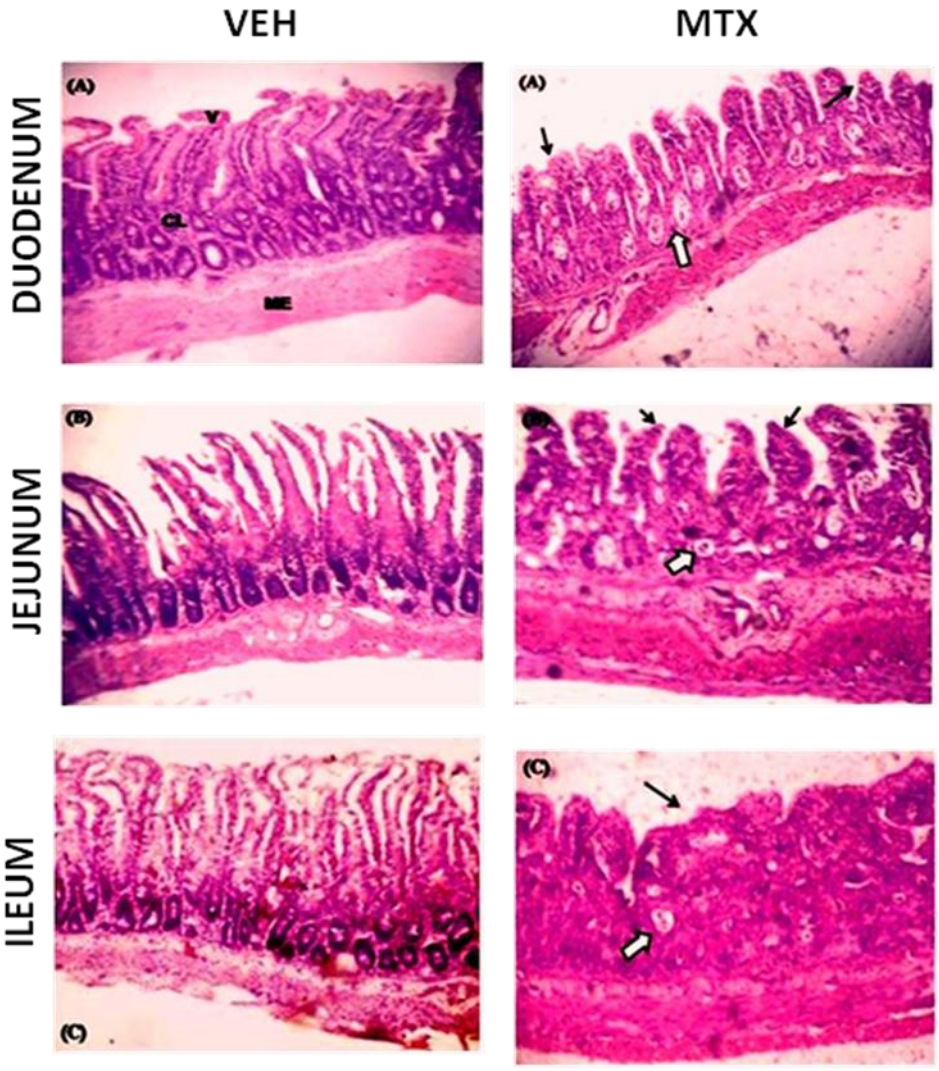
6.5.5 RESULT

(I) EFFECT OF AG PRETREATMENT ON HISTOLOGY OF MTX TREATED SMALL INTESTINE TISSUE:

AG pretreatment attenuated MTX induced small intestinal injury

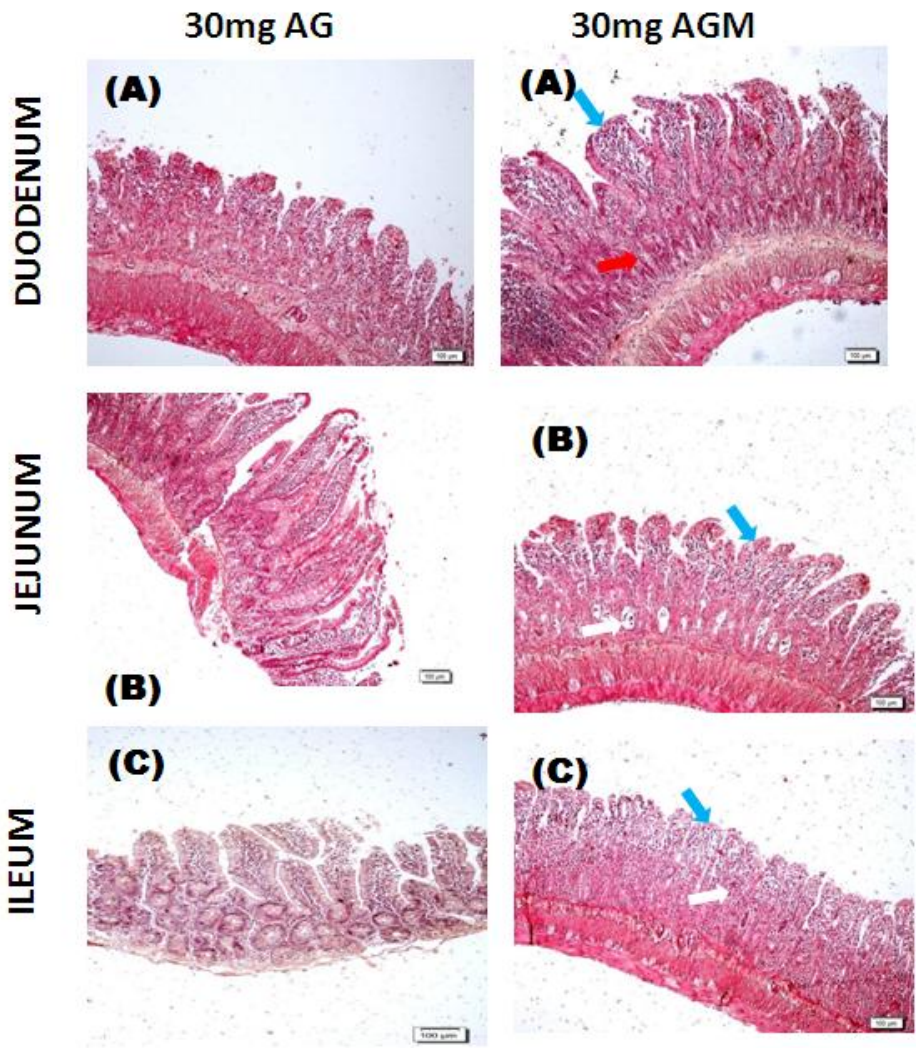
We analyzed the structure of small intestine in rats of different groups by microscopic examination As shown in Figure: 6.5.1 the architecture of mucosa under light microscopy was intact in healthy rats (control rats). The villi were present as finger-like projections and well arranged in intestinal tissue and the infiltration of inflammatory cells was hardly observed in the intestinal mucosa. Muscularis mucosae were intact at the boundary of the mucosa and submucosa in healthy rats (Figure: 6.5.1). In MTX-induced rats, massive destruction of the intestinal mucosa was observed with atrophic, shorter, and fractured villi and infiltration of inflammatory cells into the lamina propria and muscular layer. Besides, serious swell of villi and loose structure of mucous membrane were observed (Figure: 6.5.1). In contrast, AG pretreatment ameliorated MTX induced small intestinal damage in a dose-dependent manner at 30mg (Figure: 6.5.2) and 50mg (Figure: 6.5.3). After the administration of AG, mucosa morphology was significantly improved compared with MTX group. The mucosa was thicker, better organized and infiltrated with less inflammatory cells. Small intestines of rats treated with aminoguanidine alone (30 mg / 50 mg) showed normal morphology (Figure: 6.5.2 and Figure: 6.5.3). Thus AG pretreatment had a dose dependent protective effect against the histological damage induced by MTX.

Figure: 6.5.1



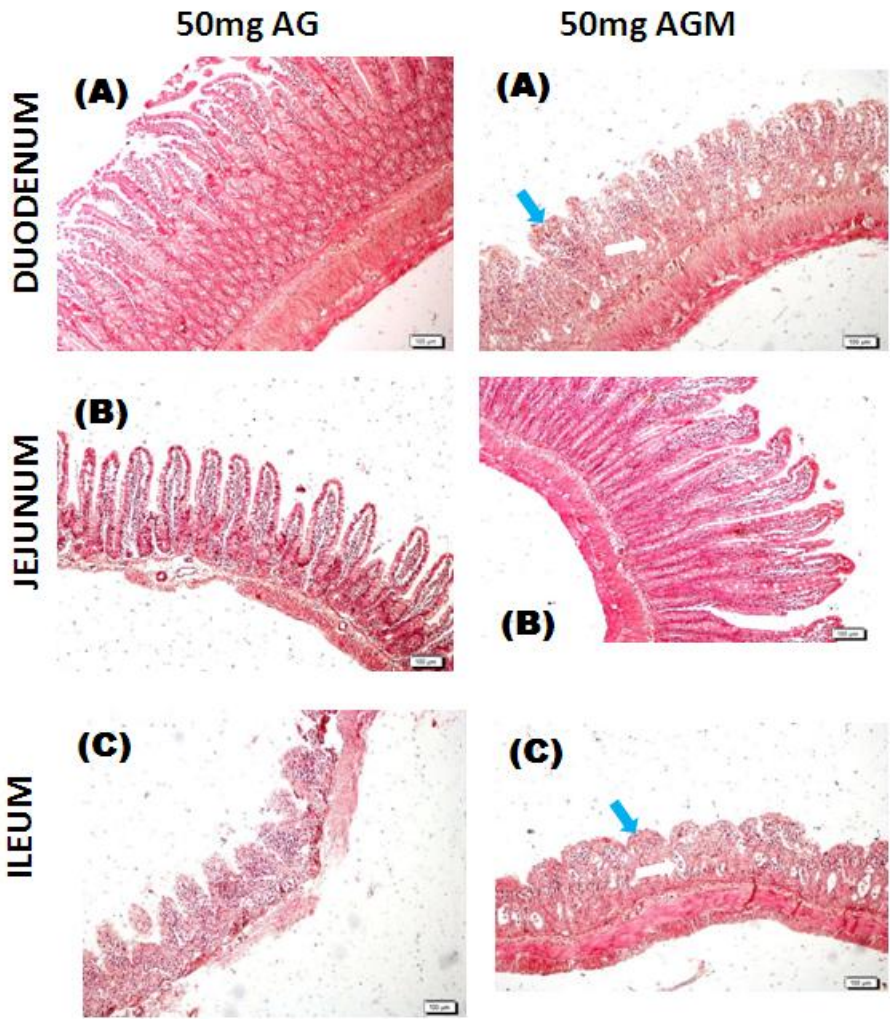
Histology of the duodenum (A), jejunum (B) and ileum (C) of control and MTX after 24 hours of final dose, magnification X100. V – Villus, CL – Crypts of Leiberkhun; ME – Muscularis Externa. Control rats showing normal architecture. MTX rats show morphological changes like, villi were shortened in the duodenum (A), distorted in the jejunum (B) and aborted, flattened, blunted and fused in the ileum (C). Black arrow indicates the villi and the white arrow indicates the crypt abscess.

Figure: 6.5.2



Histology of the duodenum (A), jejunum (B) and ileum (C) of 30mg aminoguanidine alone (30mg AG) and 30mg aminoguanidine pretreated MTX (30mg AGM) after 24 hours of final dose, magnification X100. 30mg AG rats show normal architecture. 30mg AGM rats show taller villi and normal crypt in the duodenum (A), taller villi and crypt abcess in the jejunum (B) and taller villi and crypt improvement in the ileum (C). Blue arrow indicates the normal villi, red arrow indicates normal crypt and the white arrow indicates morphological changes in the crypt.

Figure: 6.5.3



Histology of the duodenum (A), jejunum (B) and ileum (C) of 50mg aminoguanidine alone (50mg AG) and 50mg aminoguanidine pretreated MTX (50mg AGM) after 24 hours of final dose, magnification X100. 50mg AG rats show normal architecture. 50mg AGM rats show taller villi and improvement of crypt in the duodenum (A), normal architecture of villi and crypt in the jejunum (B) and taller villi and crypt abcess in the ileum (C). Blue arrow indicates the normal villi and the white arrow indicates morphological changes in the crypt.

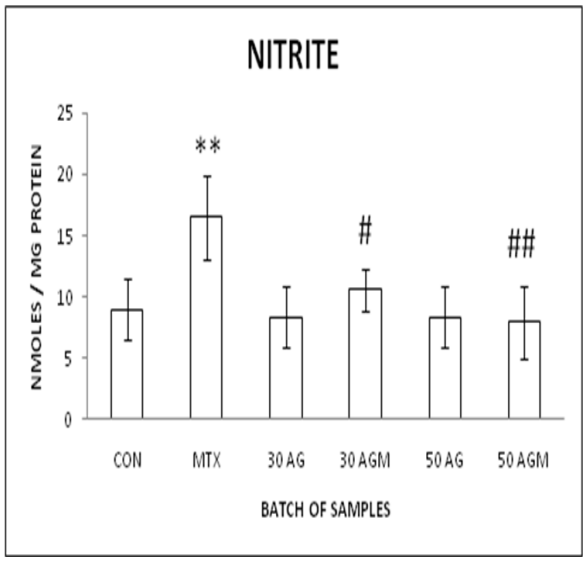
(II) EFFECT OF AG PRETREATMENT ON NITROSATIVE STRESS PARAMETER IN THE SMALL INTESTINES OF MTX TREATED RATS:

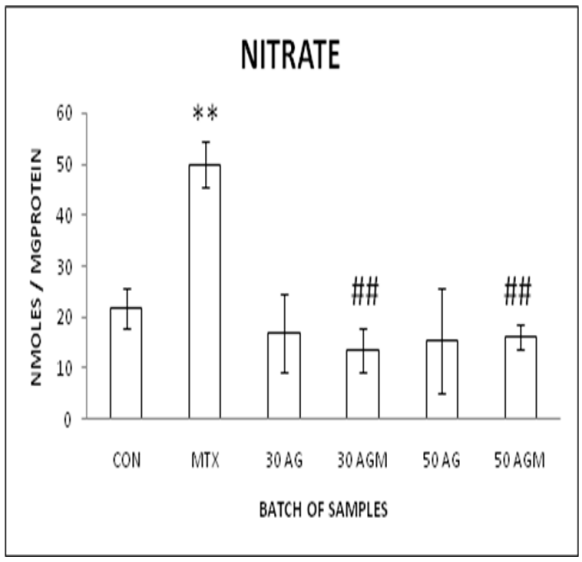
AG pretreatment reduced MTX induced increased NO level, iNOS protein expression and protein tyrosine nitration in the small intestines

MTX treatment resulted in more than 2 fold increase in NO levels (nitrite + nitrate) in the small intestines of rats. Pretreatment with AG suppressed the levels of nitric oxide more than 50% caused by MTX (Figure: 6.5.4).

With respect to iNOS immunostaining, MTX-treated rats presented intense iNOS immunostaining in the enterocytes, lamina propria cells, and neutrophils and other inflammatory cells surrounding and within necrotic crypts, when compared to the weak immunostaining of villus and crypt regions from unchallenged rats. Aminoguanidine pretreatment considerably reduced the immunostaining for iNOS (Figure: 6.5.5). iNOS mRNA levels were increased 2 fold in the small intestines of MTX treated rats as compared with control. However, AG pretreatment had no significant effect on iNOS gene expression (Figure: 6.5.6).

Rats receiving MTX presented intense immuno-labeling for nitrotyrosine both in enterocytes and lamina propria cells, in contrast to the weak immunostaining seen in the control rats. Aminoguanidine pretreatment considerably reduced the immunostaining for nitrotyrosine (Figure: 6.5.7). Thus, aminoguanidine pretreatment attenuates MTX induced nitrosative stress in the small intestines.





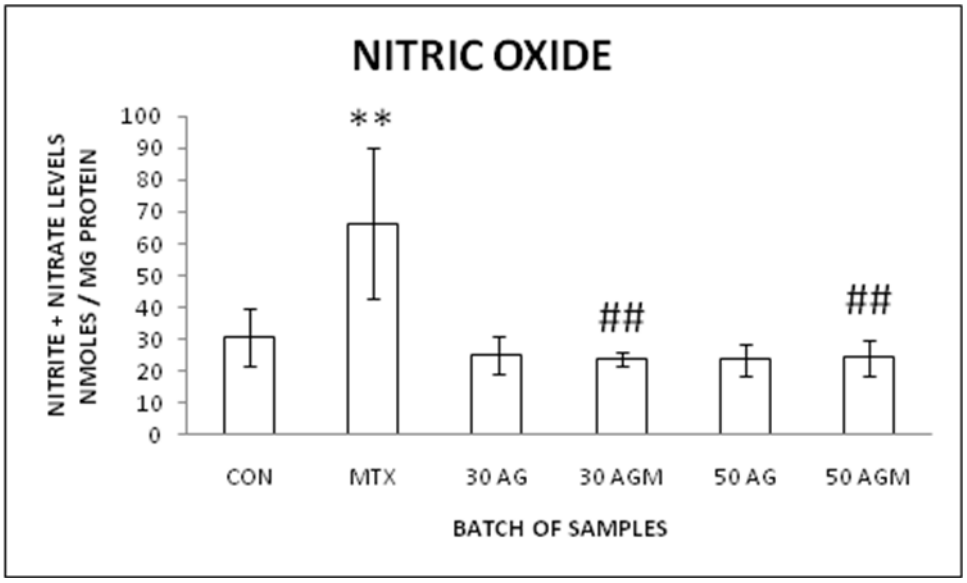
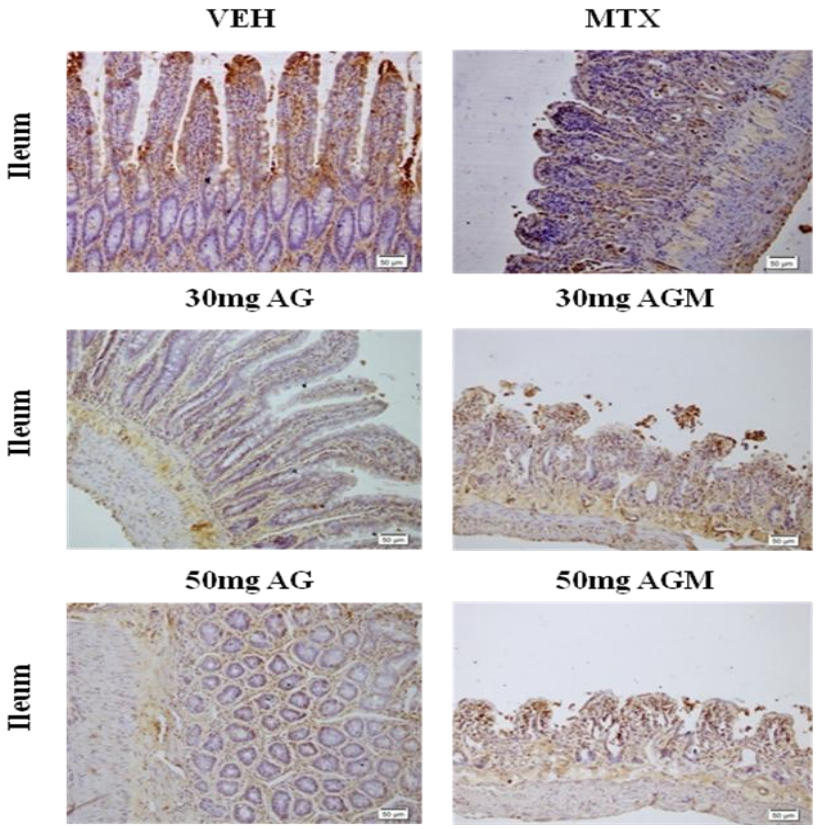


Figure: 6.5.4 – Nitrite, nitrate and nitric oxide levels in the small intestine mucosa of control and experimental rats, 24 hours after the final dose of vehicle, MTX and AG respectively. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control, # P value < 0.05 as compared to MTX, ## P value < 0.005 as compared to MTX.

Figure: 6.5.5 **iNOS IHC**



Representative image of iNOS protein expression in the ileum of rats 24 hours after the treatment with MTX and AG pretreated by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of iNOS when compared to basal immunostaining in control rat small intestine tissue treated with vehicle. The expression was decreased in AG pretreated (30mg AGM and 50mg AGM) samples when compared to MTX.

Site	VEH	MTX	30mg AG	30mg AGM	50mg AG	50mg AGM
Duodenum	+/-	++	+/-	+	+/-	+
Jejunum	+/-	++	+/-	++	+/-	+
Illeum	+	+++	+/-	++	+/-	++

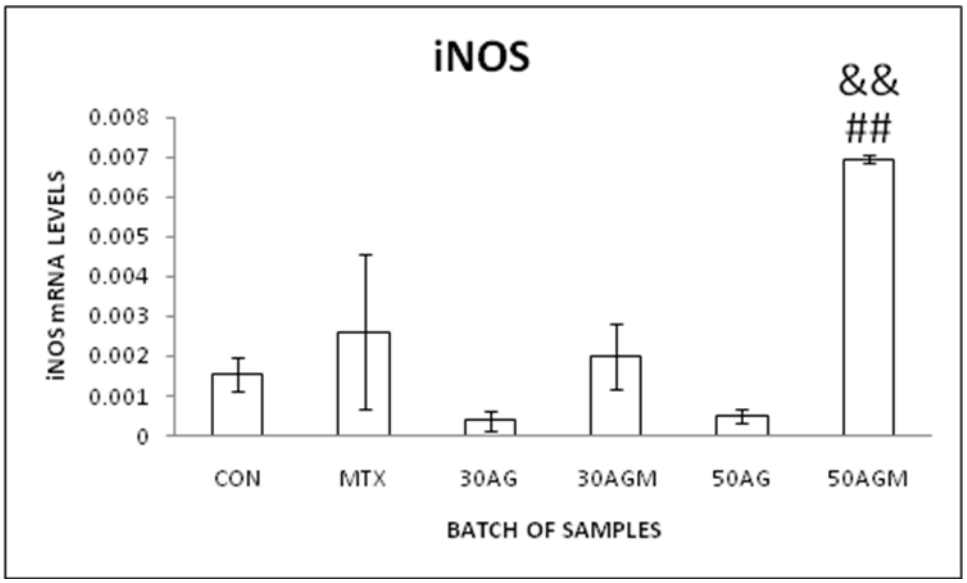
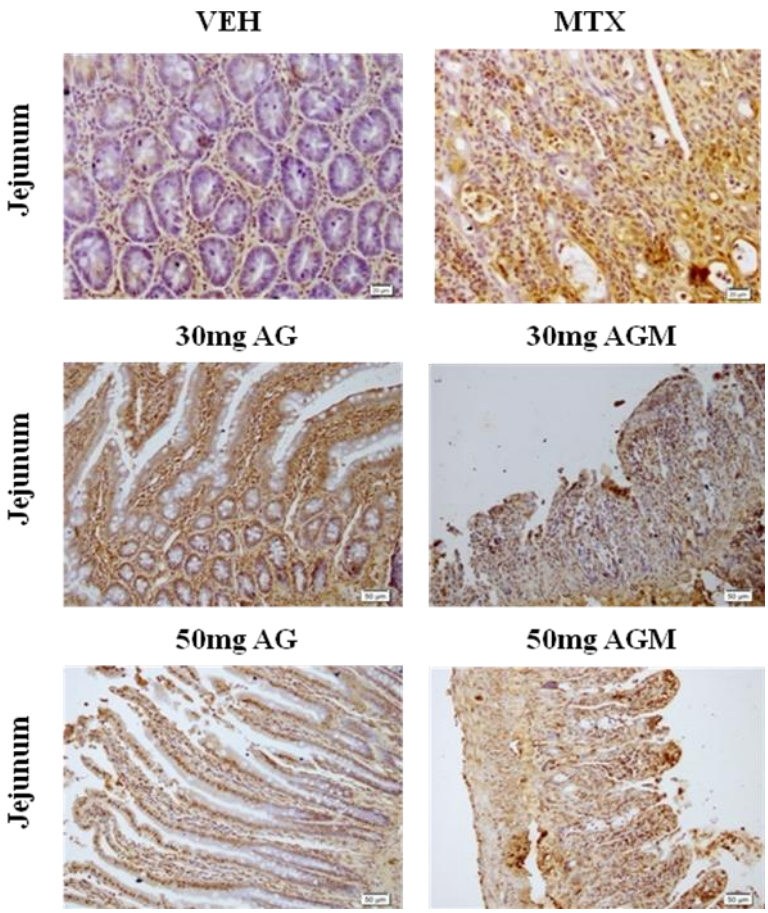


Figure: 6.5.6 - Effect of AG on MTX treated iNOS mRNA levels. RT-PCR analysis of iNOS mRNA expression after 24hours of final dose of MTX treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, ## P value < 0.005 as compared to MTX, && P value < 0.005 as compared to 30AGM.

Figure: 6.5.7 **NTY IHC**



Representative image of NTY protein expression in the jejunum of rats 24 hours after the treatment with MTX and AG pretreated by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of NTY when compared to basal immunostaining in control rat small intestine tissue treated with vehicle. The expression was decreased in AG pretreated (30mg AGM and 50mg AGM) samples when compared to MTX.

Site	VEH	MTX	30mg AG	30mg AGM	50mg AG	50mg AGM
Duodenum	+/-	++	++	+++	++	++
Jejunum	+/-	+++	++	++	+	++
Ileum	++	+++	++	++	++	+++

(III) EFFECT OF AG PRETREATMENT ON MTX INDUCED ALTERATIONS IN ENZYME ACTIVITY IN SMALL INTESTINES

AG pretreatment restored MTX induced alterations in the activities of mitochondrial ETC complexes and TCA cycle enzymes

We analyzed the activities of enzymes involved in the energy production of the cell to assess the protective role of aminoguanidine on insufficient energy metabolism caused by MTX. The MTX treatment resulted in significant reduction in the activities of ETC complexes: 60% cytochrome c oxidase - complexes IV (Figure: 6.5.8) and more than 50% F1 ATPase - complex V (Figure: 6.5.9) in the small intestine. AG pretreatment significantly restored the activities of these enzymes as compared to MTX.

The MTX treatment also resulted in significant reduction in the activities of TCA cycle enzymes and creatine kinase: 65% in aconitase (Figure: 6.5.10), 70% in succinate dehydrogenase (Figure: 6.5.11), and 80% in creatine kinase (Figure: 6.5.12) in the intestines. AG pretreatment restored the activities of all these enzymes.

AG pretreatment restored MTX induced alterations in the activities of antioxidant enzymes in the small intestines

To investigate the involvement of antioxidant enzymes in mediating antioxidant activity of AG, the activities of the intracellular antioxidant enzymes were measured in the different treatment groups. The activities of SOD, CAT, GST and CA were done. To eliminate ROS from the cellular system, SOD and CAT function coordinately to remove superoxide radicals. In the MTX-treated group, SOD activity was increased by 1.66 fold compared to the vehicle control. In the AG pretreated group, the activity was found to be decrease, which was comparable to the

MTX (Figure: 6.5.14). SOD catalyzes the dismutation of superoxide radicals into O2 and H2O2, which is in turn converted into H2O by catalase, thus preventing oxidative stress. In this study, the activity of catalase was decreased by 70% s in the MTX treated rats, and this decrease in activity was prevented in the AG pretreated rats (Figure: 6.5.15).

GST, a secondary antioxidant enzyme used in drug detoxification, helps in the radical-scavenging activity of GSH. The activity of GST was reduced 55% in the MTX treated group compared to the control group. Significant protection was observed in the AG pretreated group (Figure: 6.5.16). Therefore, the attenuation of these antioxidant enzyme activities reveals the antioxidant property of AG.

CA, is an enzyme which maintains the chemical environment of the cells and helps to prevent the damage to the cells. The MTX treatment decreased the activity of CA by 80% as compared to control group. AG pretreatment significantly restored the activity (Figure: 6.5.17).

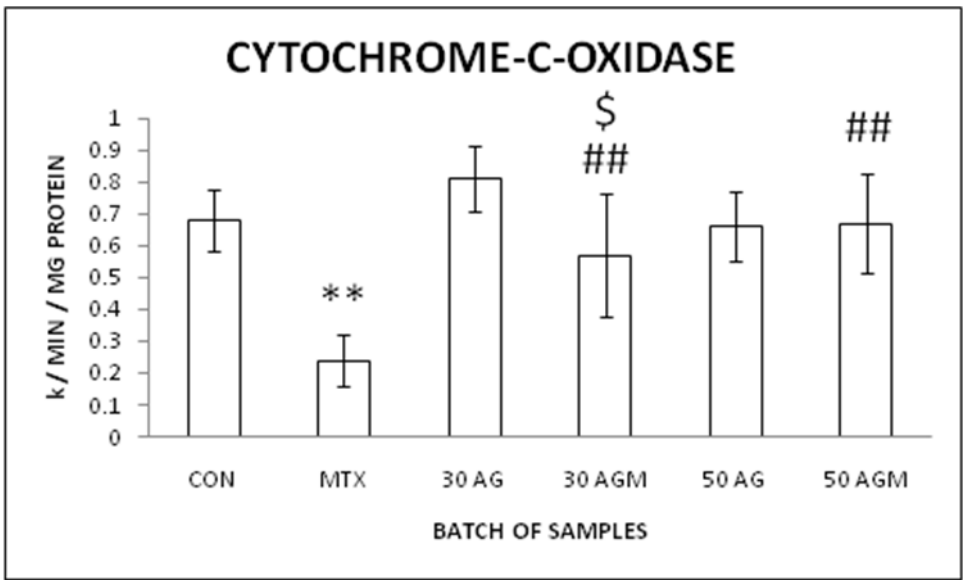


Figure: 6.5.8 - Cytochrome-C oxidase (CYTC) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control, ## P value < 0.005 as compared to MTX, $ P value < 0.05 as compared to AG.

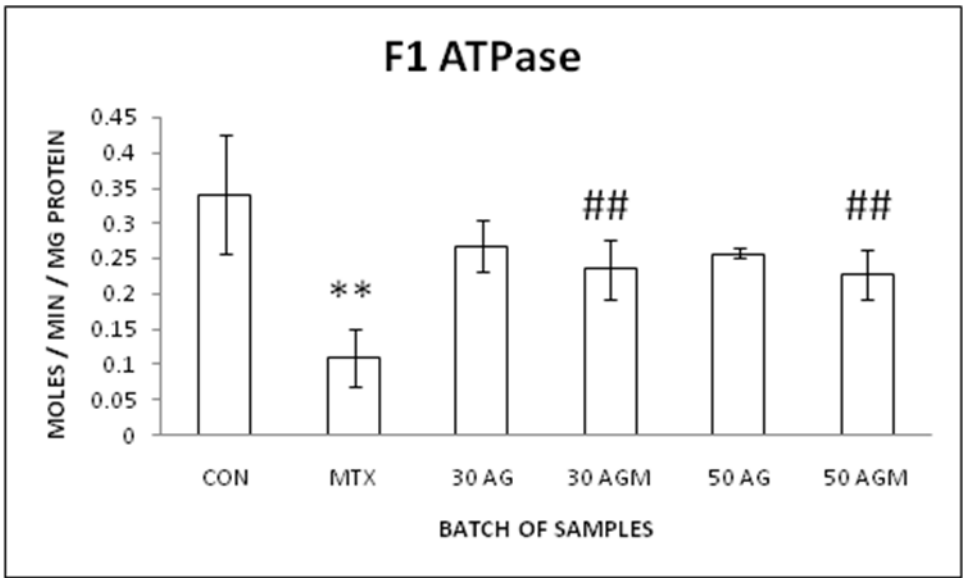


Figure: 6.5.9 - F1 ATPase (ATPase) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control, ## P value < 0.005 as compared to MTX.

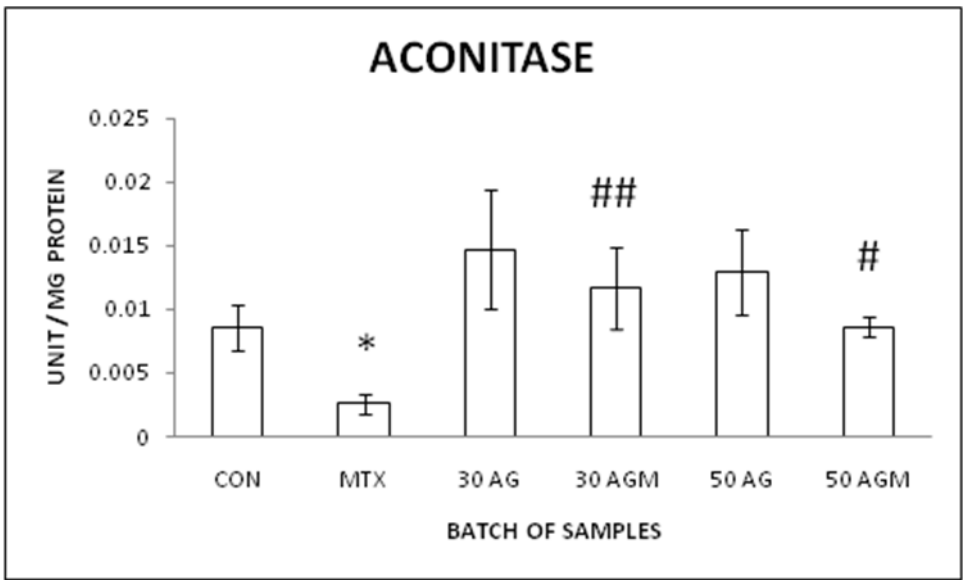


Figure: 6.5.10 - Aconitase (ACO) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control, # P value < 0.05 as compared to MTX, ## P value < 0.005 as compared to MTX.

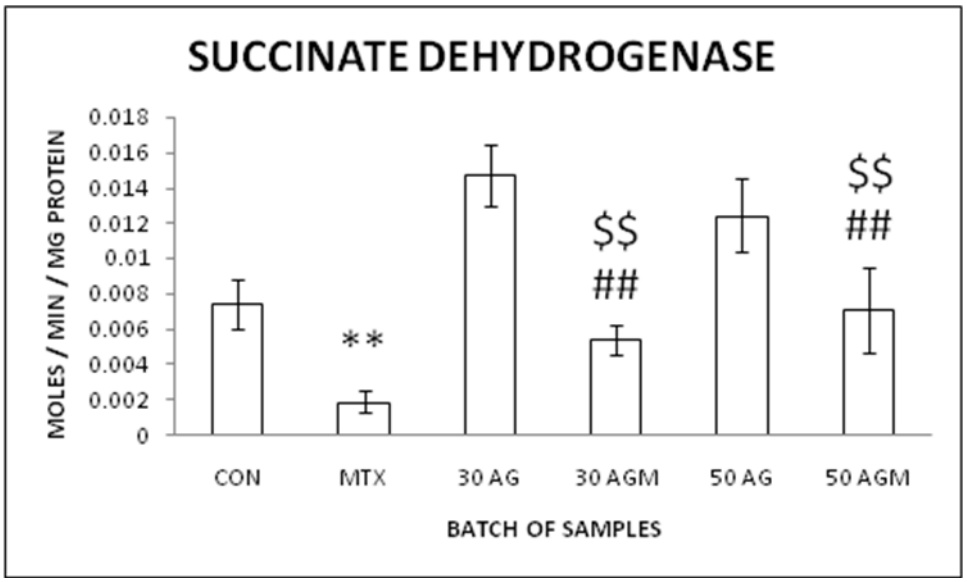


Figure: 6.5.11 - Succinate dehydrogenase (SDH) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control, ## P value < 0.005 as compared to MTX, $$ P value < 0.005 as compared to AG.

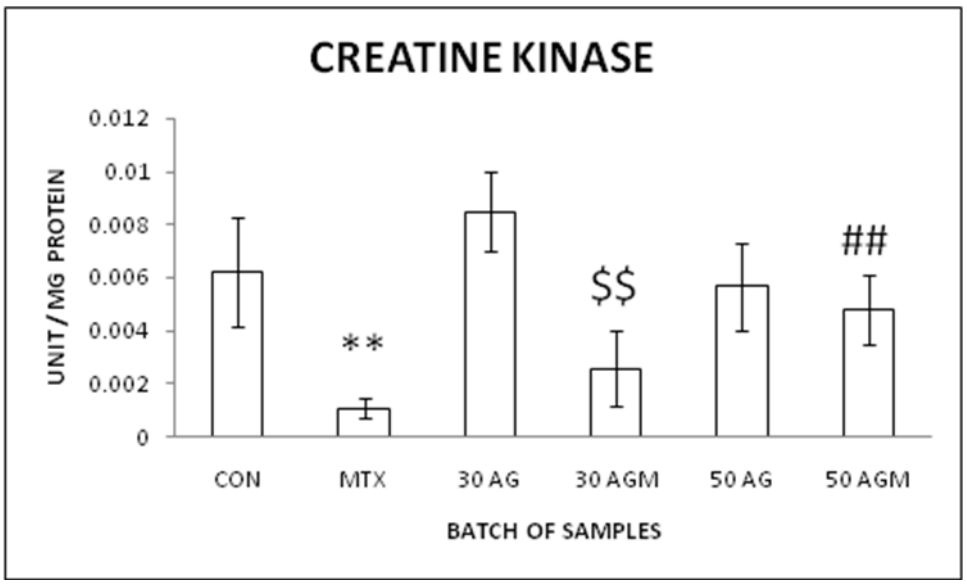


Figure: 6.5.12 - Creatine kinase (CK) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control, ## P value < 0.005 as compared to MTX, $$ P value < 0.005 as compared to AG.

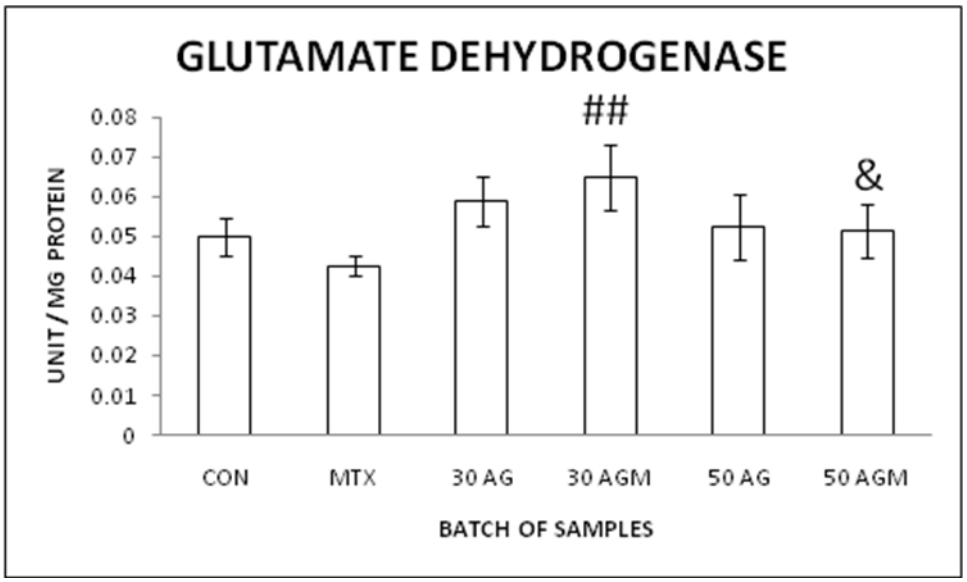


Figure: 6.5.13 - Glutamate dehydrogenase (GDH) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, ## P value < 0.005 as compared to MTX, & P value < 0.05 as compared to 30AGM.

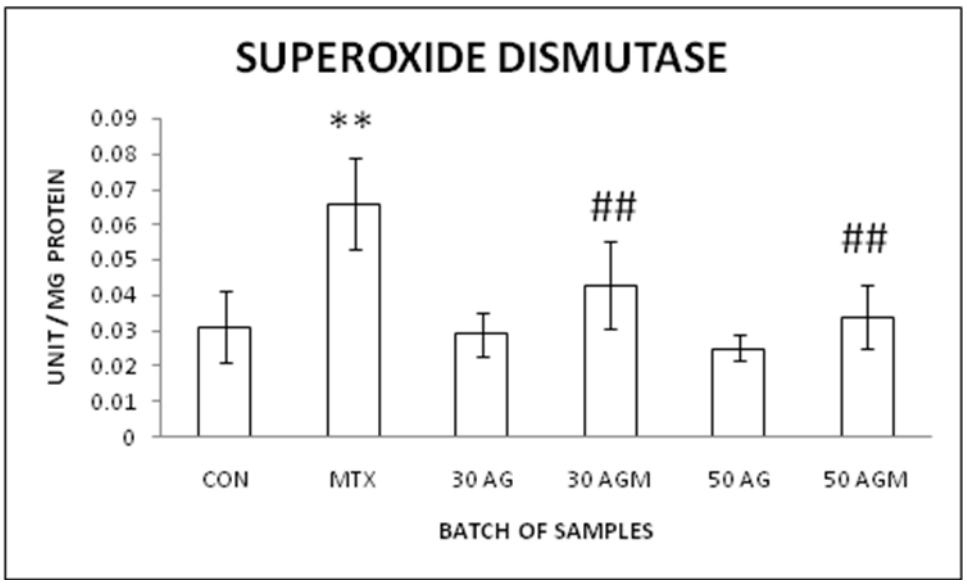


Figure: 6.5.14 - Superoxide dismutase (SOD) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control, ## P value < 0.005 as compared to MTX.

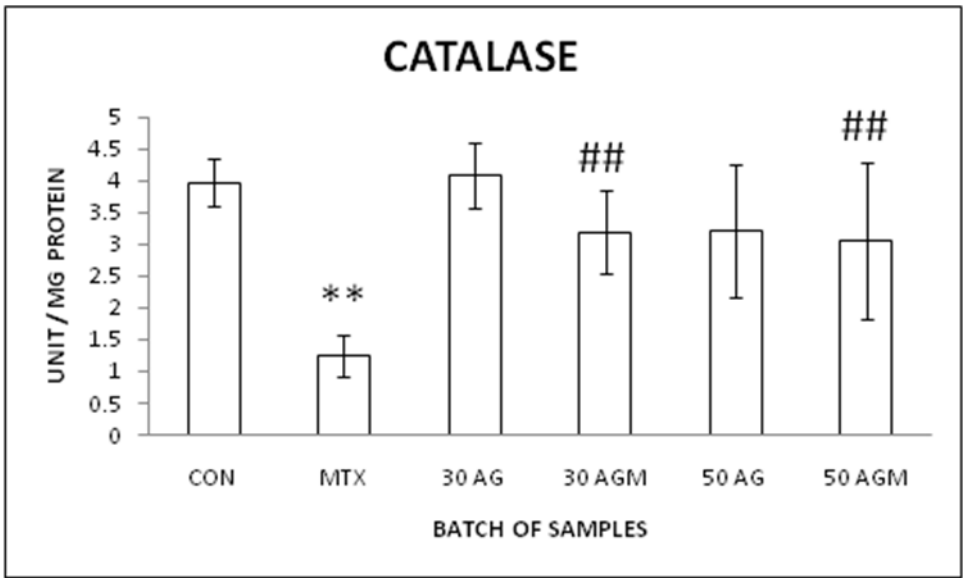


Figure: 6.5.15 - Catalase (CAT) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control, ## P value < 0.005 as compared to MTX.

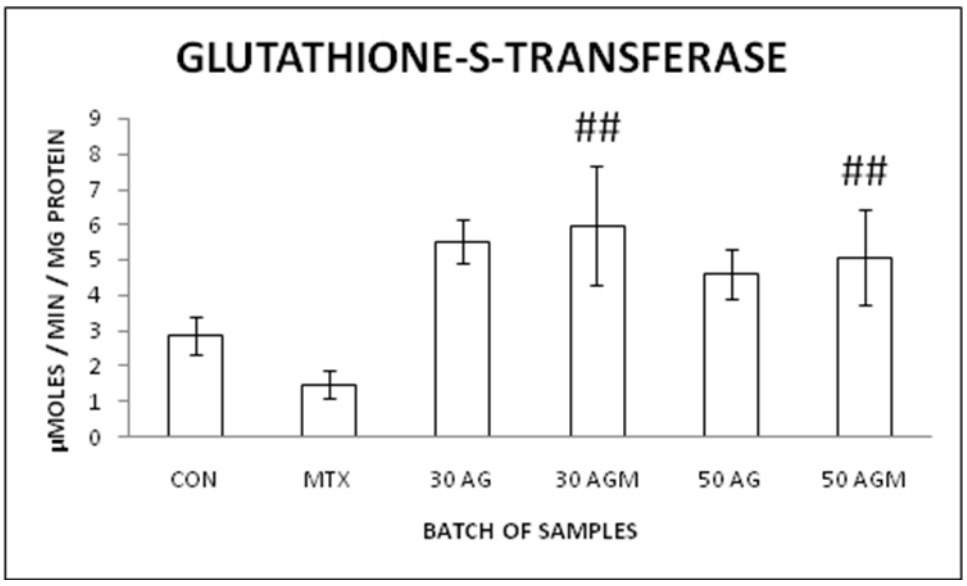


Figure: 6.5.16 - Glutathione-S-transferase (GST) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, ## P value < 0.005 as compared to MTX.

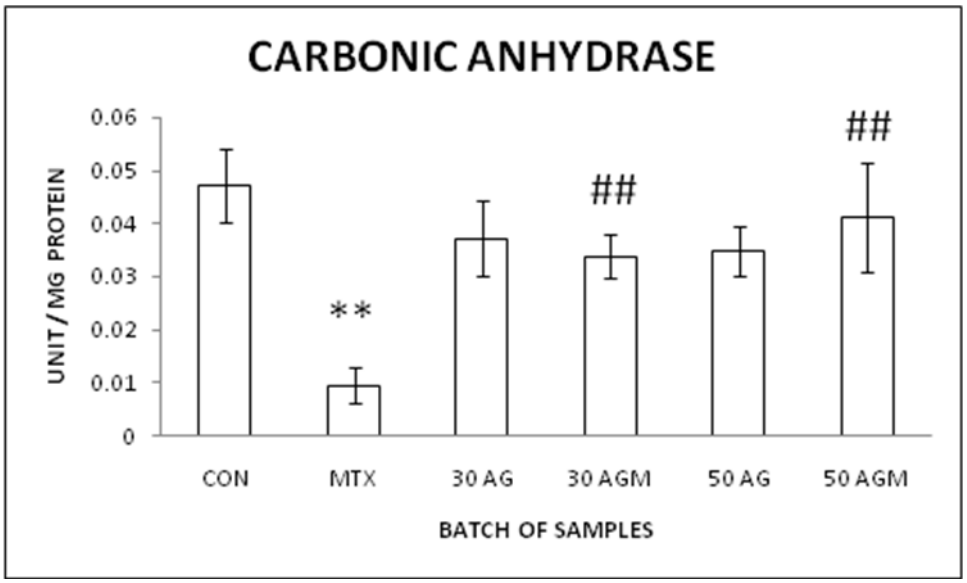


Figure: 6.5.17 - Carbonic anhydrase (CA) enzyme activity in the small intestines of control and experimental rats 24 hours after the treatment of MTX. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control, ## P value < 0.005 as compared to MTX.

(IV) EFFECT OF AG PRETREATMENT ON INFLAMMATORY SIGNALLING PATHWAY

AG pretreatment inhibited NF-κB translocation into nucleus and decreased NFκB protein level but had no effect on its gene expression

We intended to investigate whether AG pretreatment could result in inactivation of NF-κB signal pathway. We examined whether AG pretreatment could block NF-κB translocation into nucleus, since nuclear translocation seemed to correlate with NF-κB-mediated transcriptional activation. Using immunoflourescence, in the control rats, NFκB p65 was detected mainly in the cytoplasm. Treatment with MTX induced translocation of NFκB p65 from the cytoplasm to the nucleus, which was inhibited by AG pretreatment (Figure: 6.5.18).

In addition, the expression and location of NF-κB p65 was examined by immunohistochemistry in small intestinal tissue. The expression level of NF-κB p65 in MTX treated group was significantly higher than that in the control group. NF-κB p65 was mainly expressed in the nucleus, cytoplasm of epithelia. AG pretreatment significantly reduced the expression level of NF-κB compared to the MTX treated rats without AG pre treatment (Figure: 6.5.19).

AG pretreatment had no effect on MTX induced increase in NFκB mRNA expression (Figure: 6.5.20). The results indicate that AG pretreatment suppresses the inflammation response in MTX induced mucositis via NF-κB signaling pathway

AG pretreatment decreased the protein expressions of NFκB target gene: COX2 and PLA_2

With regard to COX-2 and PLA_2 protein expression the control rat intestine showed little staining whereas after treatment with MTX resulted in intense staining for COX-2 (Figure:

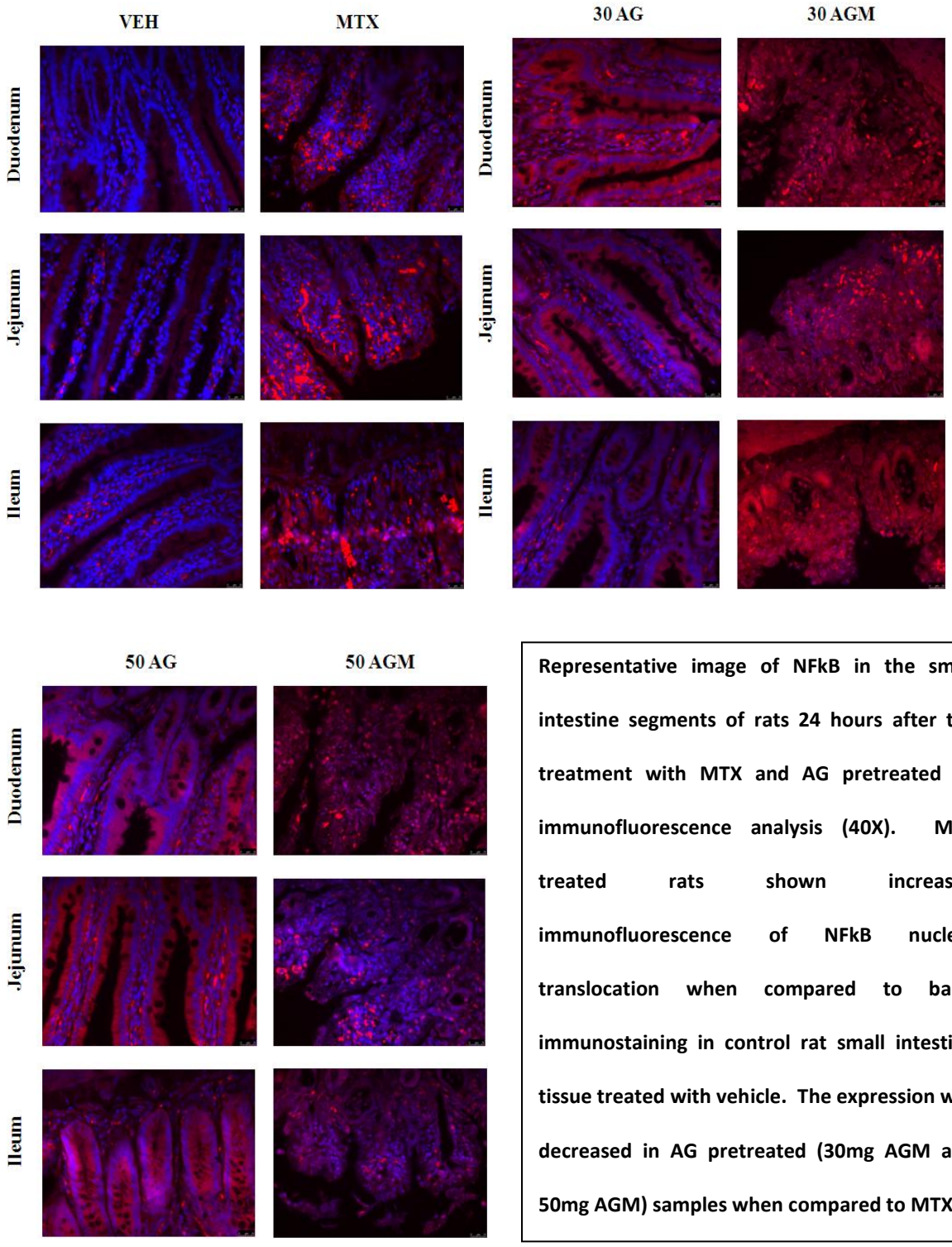
6.5.21) and PLA_2 (Figure: 6.5.22). AG pretreatment was decreased the COX-2 and PLA_2 expression as compared to MTX. The mRNA expression levels of COX-2 was in MTX treated group were significantly higher than that in the control group. However, AG pretreatment had no effect on MTX induced increase in COX-2 mRNA (Figure: 6.5.23).

AG pretreatment has no effect on gene expressions of other NFκB target genes - TNFα, MMP2, MMP9, HO-1 and HSP70

TNF-α and IL-6 are two major pro-inflammatory cytokines released in intestinal mucosa during the disruption of intestinal epithelial barrier integrity, which leads to intestinal epithelial barrier dysfunction. In order to examine the effect of AG on inflammatory response, the expression level of intestinal TNF-α was determined by RT PCR. The mRNA expression levels of TNF-α was in MTX treated group were significantly higher than that in the control group. However, AG pretreatment had no effect on MTX induced increase in TNF mRNA (Figure: 6.5.24).

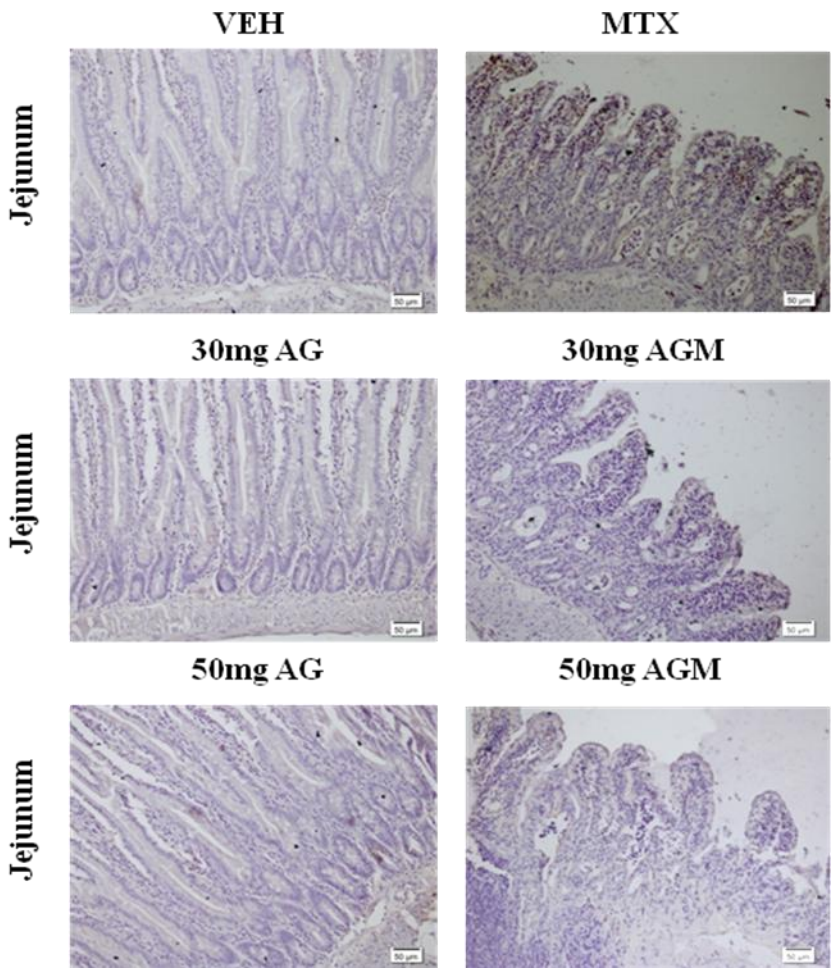
RT-PCR was performed to investigate the effect of AG in the regulation of HO-1, HSP70, MMP2, MMP9 mRNA expressions in intestinal tissue. The mRNA expressions of HO-1, HSP70, MMP2, MMP9 were significantly higher in the MTX treated groups as compared with control. AG pretreatment further increased HSP70 mRNA as compared with MTX (Figure: 6.5.26). This may be an adaptive response. However, AG pretreatment had no significant effect on MTX induced alteration in these genes HO-1 (Figure: 6.5.25), MMP2 (Figure: 6.5.27), MMP9 (Figure: 6.5.28). These results suggest that aminoguanidine exerts its beneficial effects at the translational levels and has no effect at transcription level.

Figure: 6.5.18 NFκB translocation in nucleus by IF



Representative image of NFkB in the small intestine segments of rats 24 hours after the treatment with MTX and AG pretreated by immunofluorescence analysis (40X). MTX treated rats shown increased immunofluorescence of NFkB nuclear translocation when compared to basal immunostaining in control rat small intestine tissue treated with vehicle. The expression was decreased in AG pretreated (30mg AGM and 50mg AGM) samples when compared to MTX.

Figure: 6.5.19 **NFκB IHC**



Representative image of NFκB protein expression in the jejunum of rats 24 hours after the treatment with MTX and AG pretreated by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of NFκB when compared to basal immunostaining in control rat small intestine tissue treated with vehicle. The expression was decreased in AG pretreated (30mg AGM and 50mg AGM) samples when compared to MTX.

Site	VEH	MTX	30mg AG	30mgAGM	50mg AG	50mgAGM
Duodenum	-	++	-	+	-	-
Jejunum	-	++	-	+	-	-
Illeum	-	++	-	+	-	-

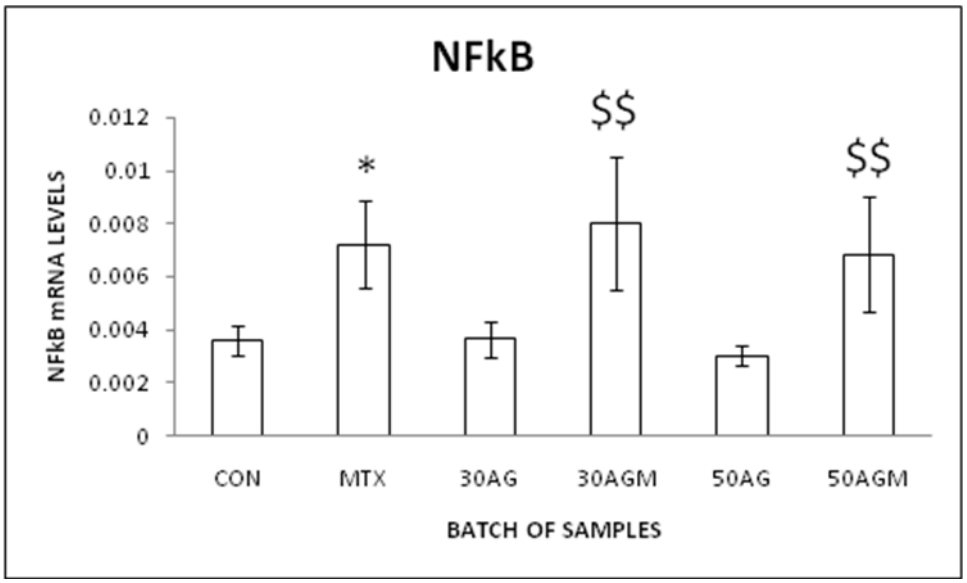
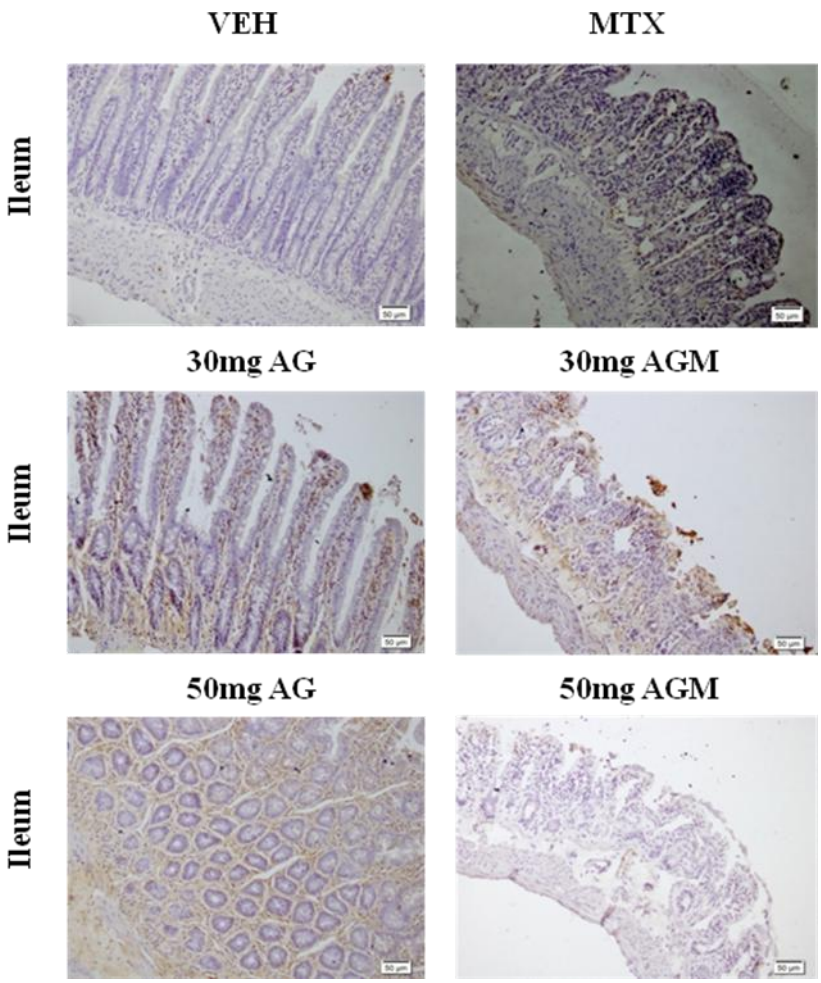


Figure: 6.5.20 - Effect of AG on MTX treated NFκB mRNA levels. RT-PCR analysis of NFκB mRNA expression after 24hours of final dose of MTX treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control, $$ P value < 0.005 as compared to AG.

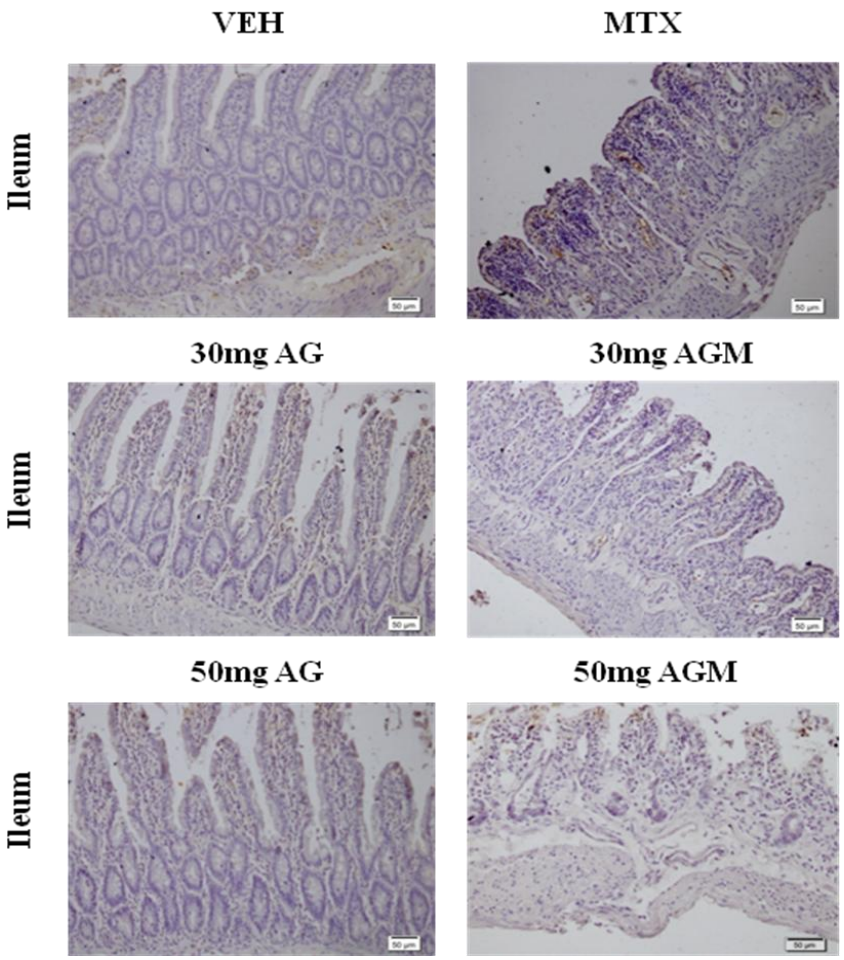
Figure: 6.5.21 **COX-2 IHC**



Representative image of COX2 protein expression in the illeum of rats 24 hours after the treatment with MTX and AG pretreated by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of COX2 when compared to basal immunostaining in control rat small intestine tissue treated with vehicle. The expression was decreased in AG pretreated (30mg AGM and 50mg AGM) samples when compared to MTX.

Site	VEH	MTX	30mg AG	30mgAGM	50mg AG	50mgAGM
Duodenum	-	+	+	+	++	+
Jejunum	-	+	+	++	+	+/-
Ileum	-	++	+	++	+/-	+

Figure: 6.5.22 **PLA_2 IHC**



Representative image of PLA_2 protein expression in the ileum of rats 24 hours after the treatment with MTX and AG pretreated by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of PLA_2 when compared to basal immunostaining in control rat small intestine tissue treated with vehicle. The expression was decreased in AG pretreated (30mg AGM and 50mg AGM) samples when compared to MTX.

Site	VEH	MTX	30mg AG	30mgAGM	50mg AG	50mgAGM
Duodenum	-ve	+	+	+	++	+
Jejunum	-ve	+	+	++	+	+/-
Ileum	-ve	++	+/-	+	+/-	+

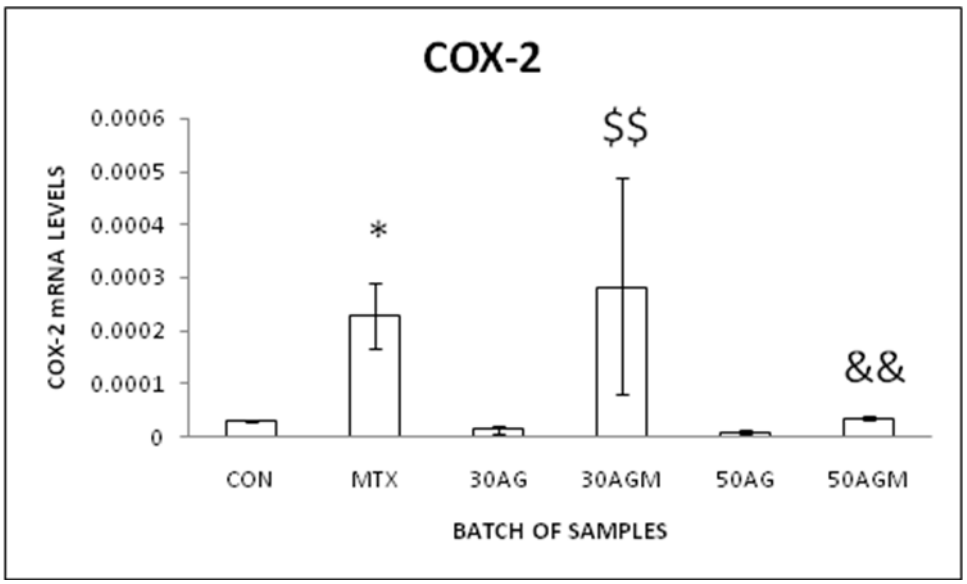


Figure: 6.5.23 - Effect of AG on MTX treated COX2 mRNA levels. RT-PCR analysis of COX2 mRNA expression after 24hours of final dose of MTX treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control, $$ P value < 0.005 as compared to AG, && P value < 0.005 as compared to 30AGM.

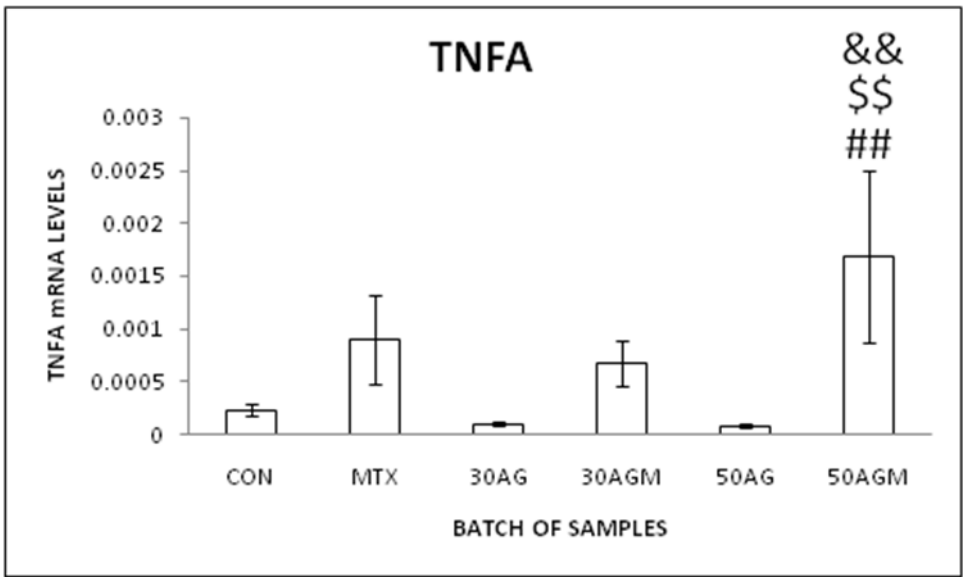


Figure: 6.5.24 - Effect of AG on MTX treated TNFα mRNA levels. RT-PCR analysis of TNFα mRNA expression after 24hours of final dose of MTX treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, ## P value < 0.005 as compared to MTX, $$ P value < 0.005 as compared to MTX, && P value < 0.005 as compared to 30AGM.

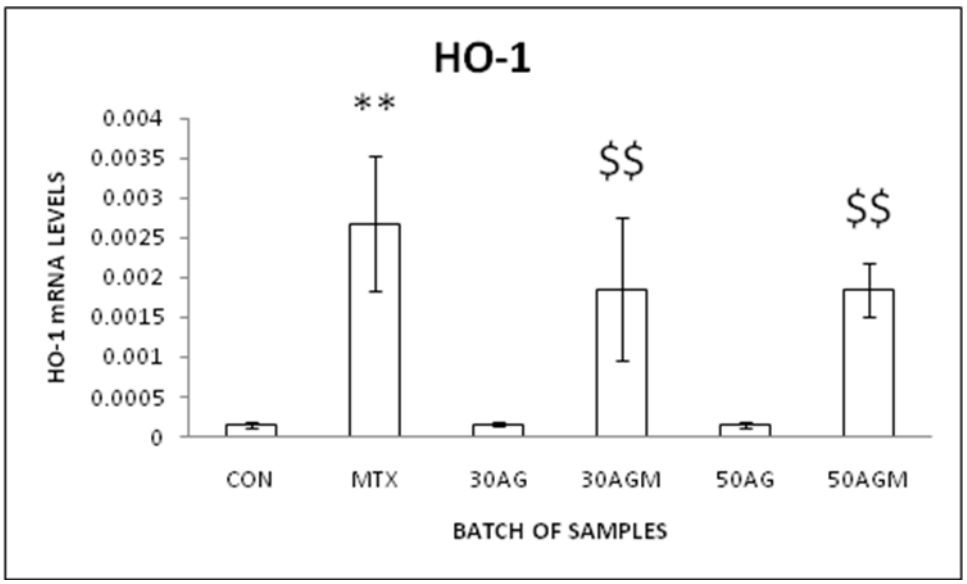


Figure: 6.5.25 - Effect of AG on MTX treated HO-1 mRNA levels. RT-PCR analysis of HO-1 mRNA expression after 24hours of final dose of MTX treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, ** P value < 0.05 as compared to control, $$ P value < 0.005 as compared to AG.

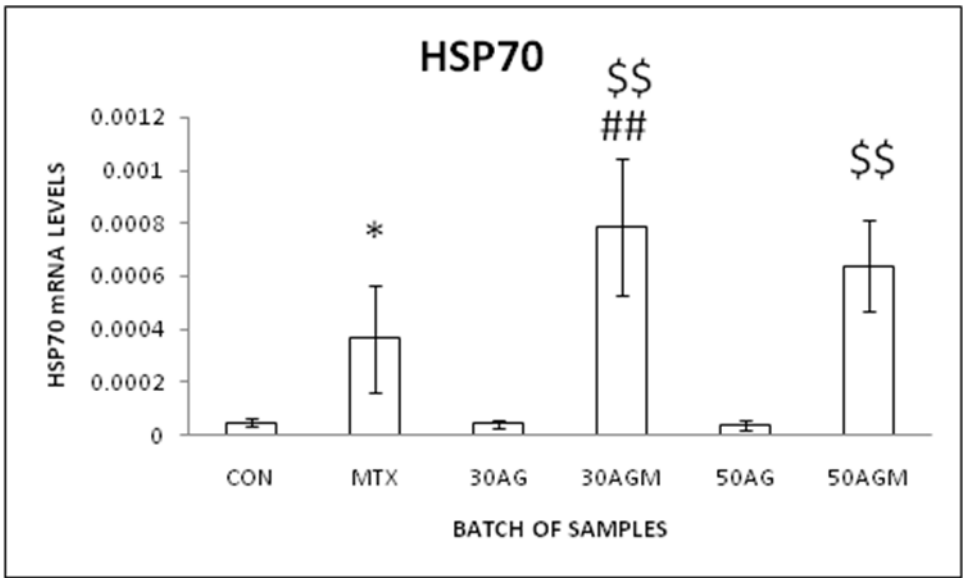


Figure: 6.5.26 - Effect of AG on MTX treated HSP70 mRNA levels. RT-PCR analysis of HSP70 mRNA expression after 24hours of final dose of MTX treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control, ## P value < 0.005 as compared to MTX, $$ P value < 0.005 as compared to 30AGM.

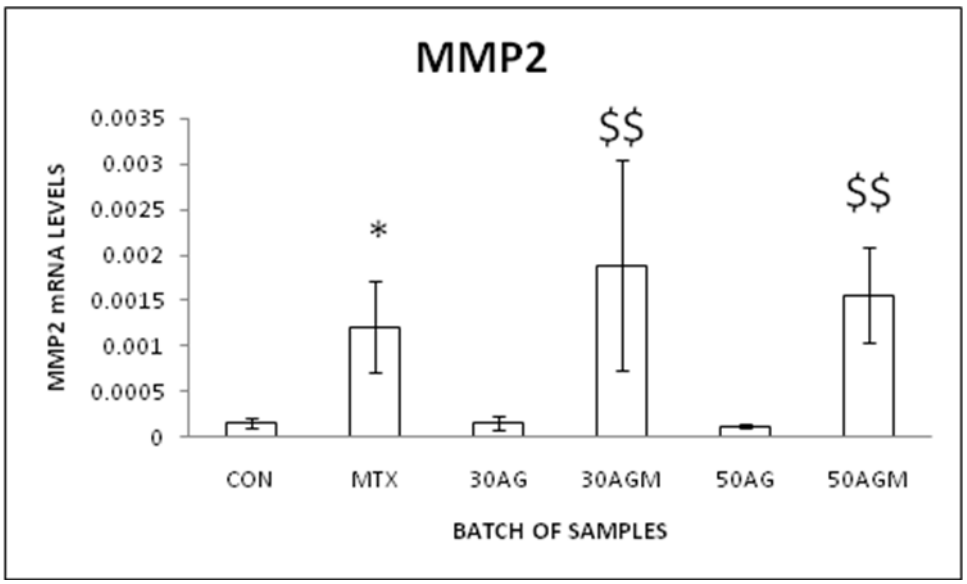


Figure: 6.5.27 - Effect of AG on MTX treated MMP2 mRNA levels. RT-PCR analysis of MMP2 mRNA expression after 24hours of final dose of MTX treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control, $$ P value < 0.005 as compared to AG.

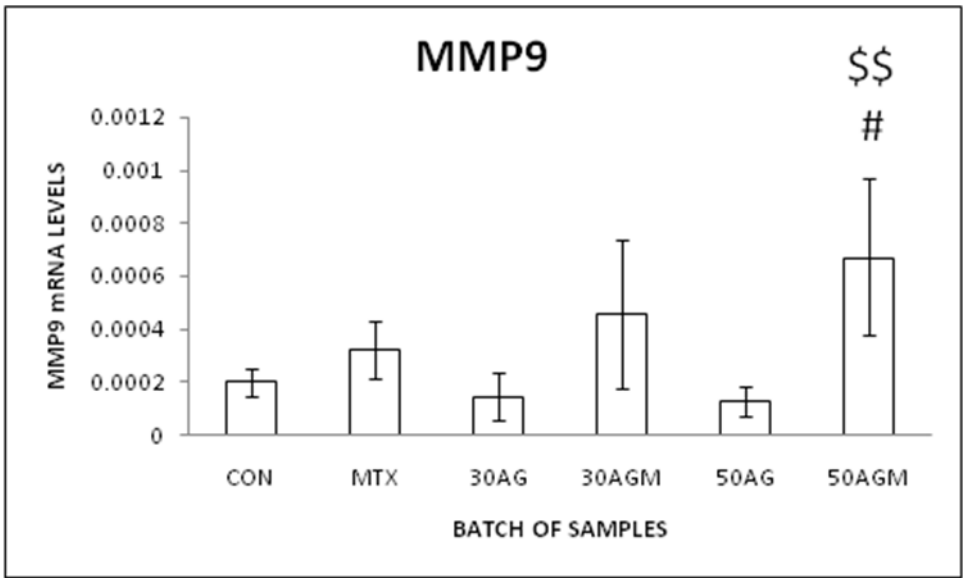


Figure: 6.5.28 - Effect of AG on MTX treated MMP9 mRNA levels. RT-PCR analysis of MMP9 mRNA expression after 24hours of final dose of MTX treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, # P value < 0.05 as compared to MTX, $$ P value < 0.005 as compared to AG.

(V) EFFECT OF AG PRETREATMENT ON APOPTOSIS SIGNALLING PATHWAY OF MTX TREATED SMALL INTESTINE TISSUE:

Disruption of the mitochondrial membrane potential and an increasing concentration of cytosolic cytochrome c are important biomarkers of oxidative stress-induced cell damage, and the subsequent activation of caspase 9/3 represents a key step in the mitochondria-dependent cell death pathway. To determine if AG pretreatment protects against the MTX induced mitochondria-mediated toxicity, critical events of the cell death pathway, including the immunoreactive concentration of caspases, PARP activation, and DNA fragmentation were measured by IHC.

Mitochondrial membrane permeability transition facilitates release of cytochrome c from mitochondria to cytosol. IHC analysis of intestines of MTX treated rats showed significant increase in cytosolic cytochrome c as compared to control, whereas in AG pretreated group this release was reduced (Figure: 6.5.29).

Cytochrome c stimulates the cytosolic assembly of the apoptosome, which leads to the oligomerization and activation of caspase 9 and cleavage of caspase 3. Furthermore, caspase 9/3 is the most frequently activated death proteases for chromatin condensation and DNA fragmentation, which are essential processes associated with the degradation of cellular machinery (447).

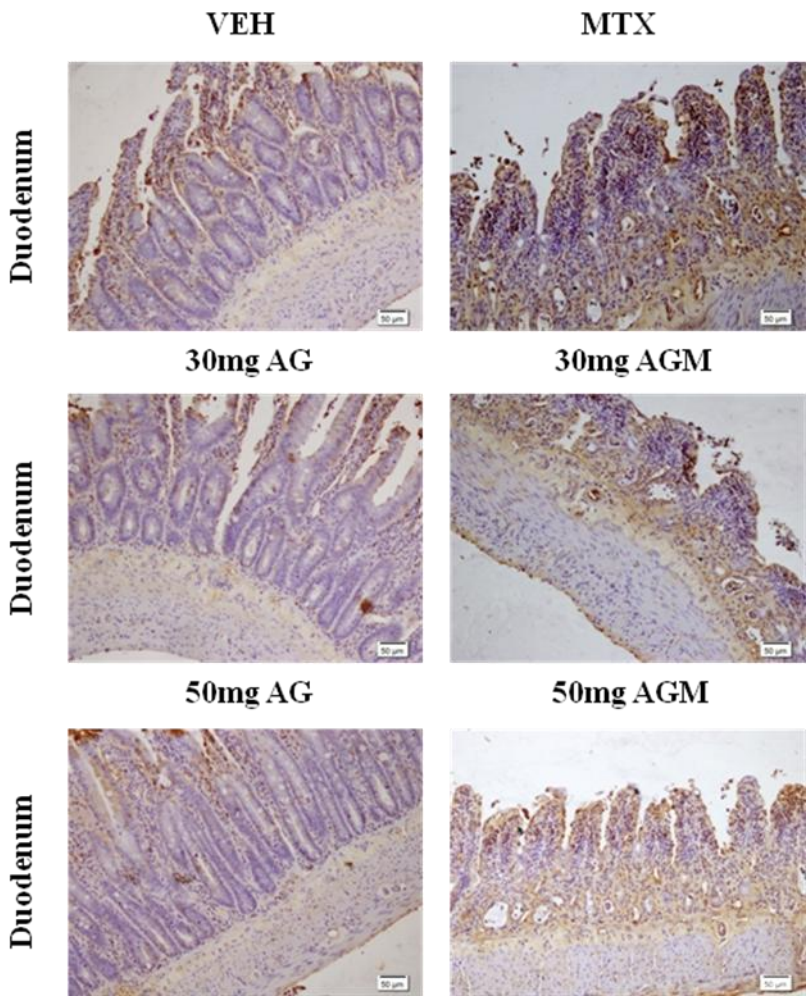
Caspase activation and PARP cleavage are hallmarks of apoptosis. Therefore, we investigated the of AG pretreatment on caspase activation and PARP cleavage. Immunohistochemistry (IHC)

for active caspases 9 (Figure: 6.5.30) and caspases 3 (Figure: 6.5.31) showed basal expression in the small intestines of control rats. In the MTX treated rat intestines, the staining for caspases was more intense and several villi enterocytes showed increased staining for caspases 9 and 3. AG pretreatment reduced MTX induced caspase activation as evidenced by weak staining for the caspases. AG pretreatment had no significant effect on MTX induced lowering of caspase 3 mRNA expression (Figure: 6.5.32).

With regard to PARP, intense staining was observed both the by immunohistochemically (Figure: 6.5.33) and immunofluorescence (Figure: 6.5.34) small intestine sections of MTX treated group. Weak immunostaining of intestinal PARP was evident in control rats. In the rats treated with MTX there was intense staining for PARP. The immunolocalisation of PARP protein expression was markedly decreased in aminoguanidine pretreated groups (30mg AGM and 50mg AGM) as compared to MTX treated group. These results suggest that AG can also suppress the intestinal PARP-1 activation triggered by MTX administration.

To determine if AG pretreatment protects against the MTX-induced DNA damage, DNA damage was qualitatively assessed through agarose gel electrophoresis. Orderly degraded DNA and the characteristic oligonucleosome length of the DNA cleavage product were observed in the MTX-treated rats. AG preatment prevented DNA fragmentation substantially as evident by the absence of DNA laddering pattern. There was no DNA fragmentation in control, 30AG and 50AG group of samples. We also found restoration of the DNA quantity in 30AG and 50AG pretreated samples as compared to MTX (Figure: 6.5.35). These results suggest that aminoguanidine pretreatment reduces apoptosis in MTX treated samples.

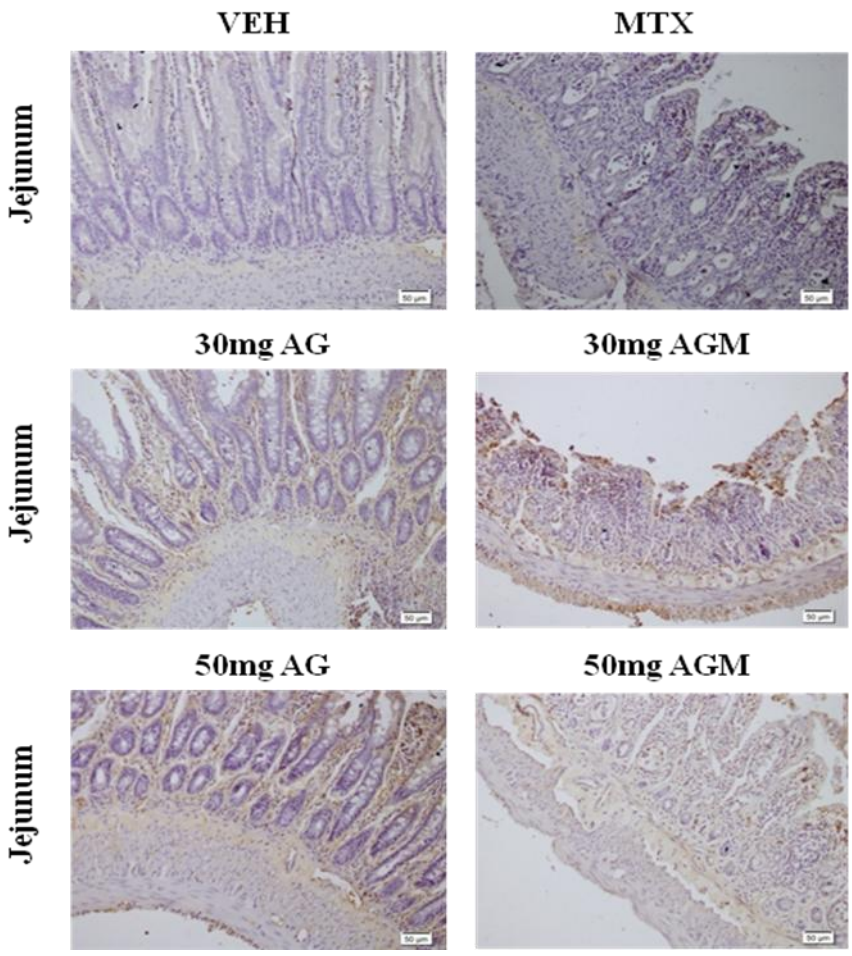
Figure: 6.5.29 **CYT-C IHC**



Representative image of CYTC protein expression in the duodenum of rats 24 hours after the treatment with MTX and AG pretreated by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of CYTC when compared to basal immunostaining in control rat small intestine tissue treated with vehicle. The expression was decreased in AG pretreated (30mg AGM and 50mg AGM) samples when compared to MTX.

Site	VEH	MTX	30mg AG	30mgAGM	50mg AG	50mgAGM
Duodenum	+	+++	+	++	+	++
Jejunum	+/-	+++	+	++	+	++
Illeum	+	+++	+	++	+	++

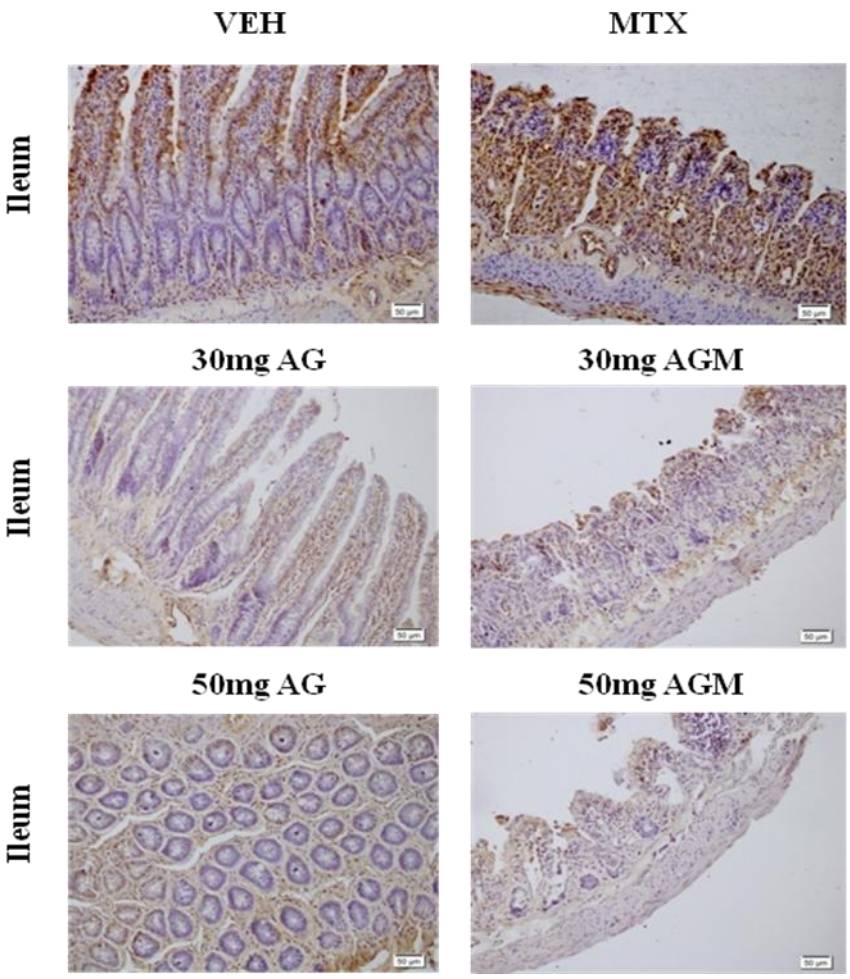
Figure: 6.5.30 **CASP-9 IHC**



Representative image of CASP9 protein expression in the jejunum of rats 24 hours after the treatment with MTX and AG pretreated by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of CASP9 when compared to basal immunostaining in control rat small intestine tissue treated with vehicle. The expression was decreased in AG pretreated (30mg AGM and 50mg AGM) samples when compared to MTX.

Site	VEH	MTX	30mg AG	30mgAGM	50mg AG	50mgAGM
Duodenum	+	+++	+	++	+	+
Jejunum	+	+++	+	++	+	+
Illeum	+	+++	+	++	+	+

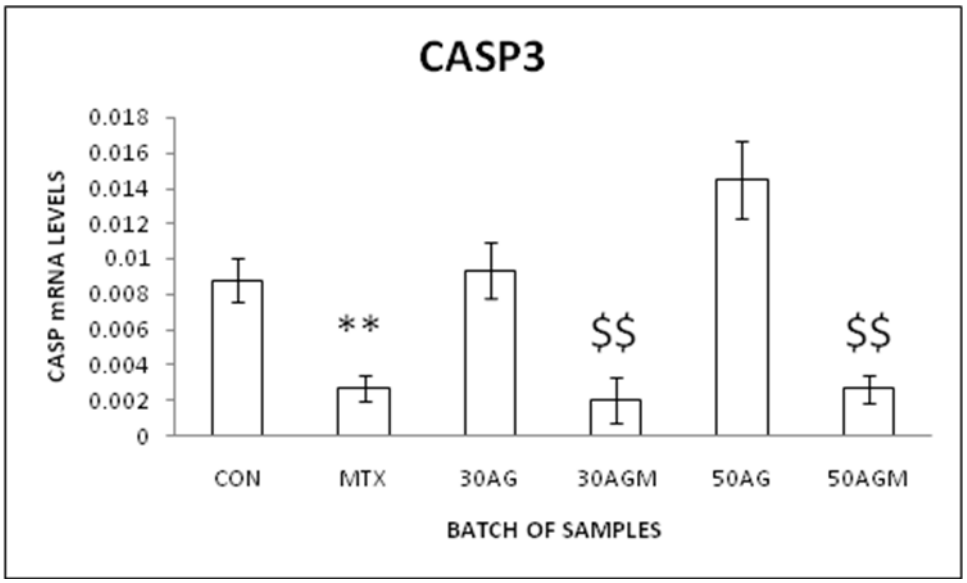
Figure: 6.5.31 **CASP-3 IHC**



Representative image of CASP3 protein expression in the ileum of rats 24 hours after the treatment with MTX and AG pretreated by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of CASP3 when compared to basal immunostaining in control rat small intestine tissue treated with vehicle. The expression was decreased in AG pretreated (30mg AGM and 50mg AGM) samples when compared to MTX.

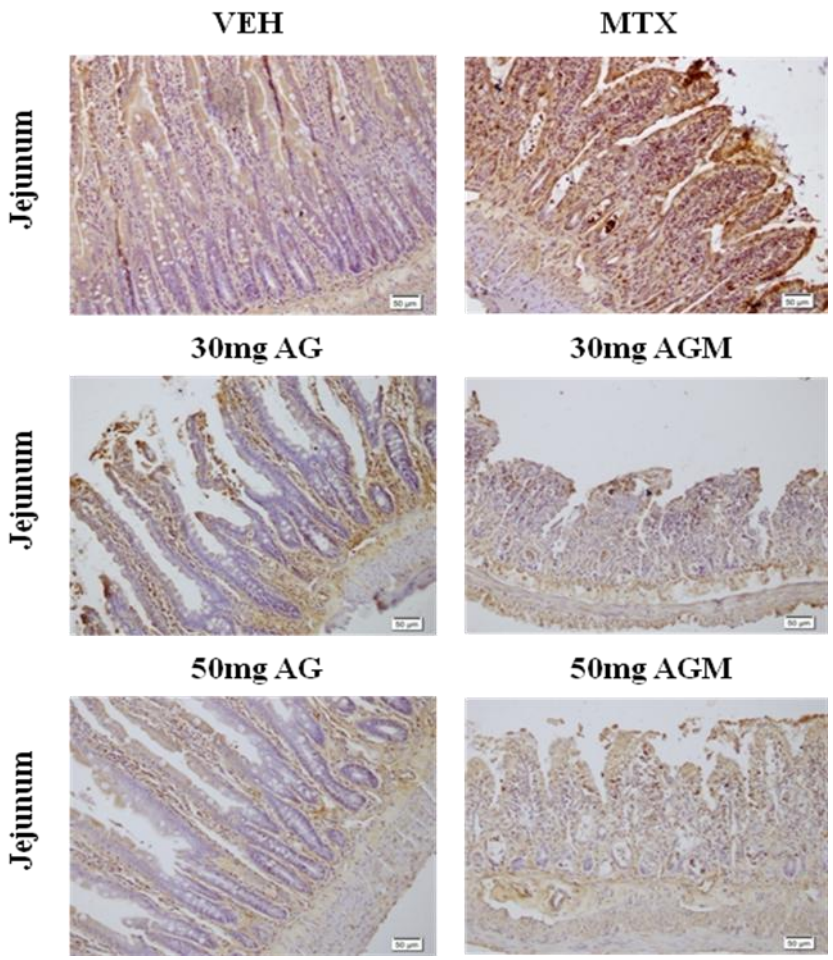
Site	VEH	MTX	30mg AG	30mgAGM	50mg AG	50mgAGM
Duodenum	+/-	++	+	++	++	++
Jejunum	+	+++	+	++	+	++
illeum	+	+++	+	++	+	++

Figure: 6.5.32



Effect of AG on MTX treated CASP3 mRNA levels. RT-PCR analysis of CASP3 mRNA expression after 24hours of final dose of MTX treated small intestine mucosa tissue. Data represent mean ± SD, N = 6 in each group, ** P value < 0.005 as compared to control, $$ P value < 0.005 as compared to AG.

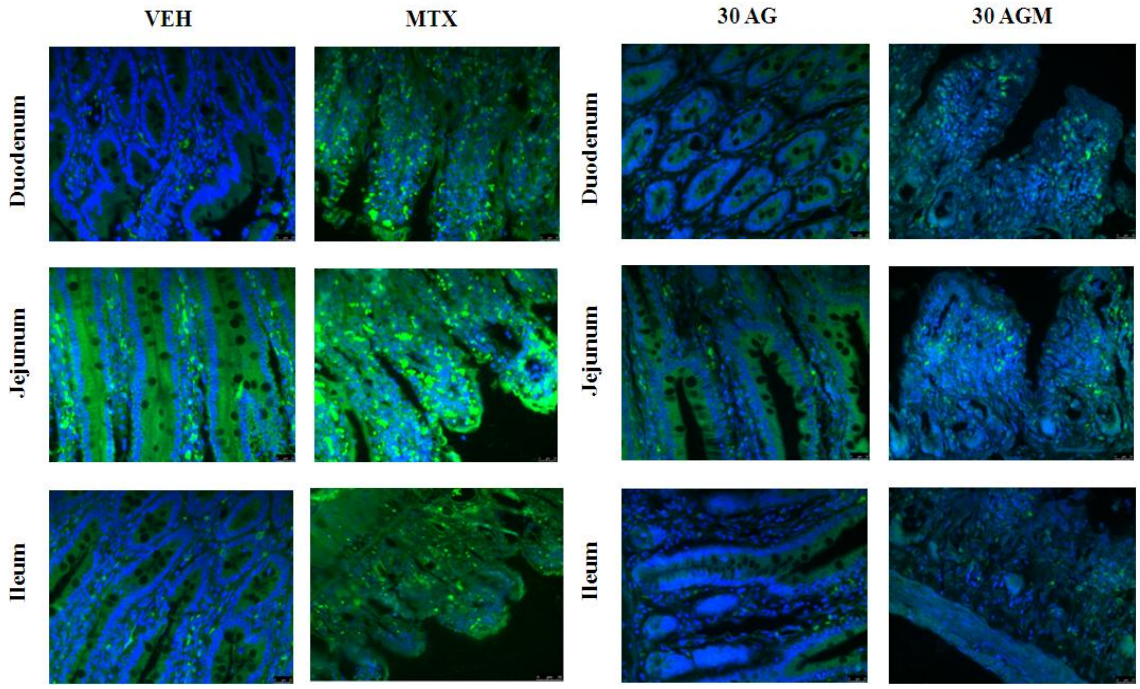
Figure: 6.5.33 **PARP IHC**

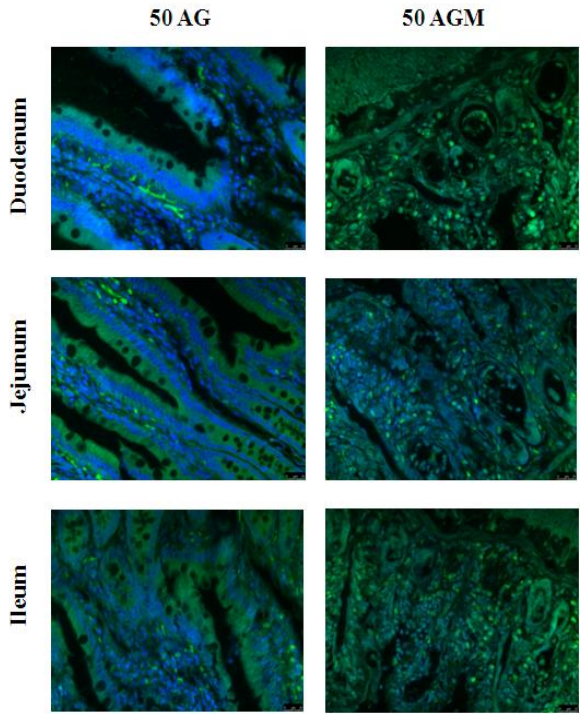


Representative image of PARP protein expression in the jejunum of rats 24 hours after the treatment with MTX and AG pretreated by immunohistochemical analysis (20X). MTX treated rats shown increased immunostaining of PARP when compared to basal immunostaining in control rat small intestine tissue treated with vehicle. The expression was decreased in AG pretreated (30mg AGM and 50mg AGM) samples when compared to MTX.

Site	VEH	MTX	30mg AG	30mgAGM	50mg AG	50mgAGM
Duodenum	+	++	+	++	+	++
Jejunum	-	+++	+	+	+	+
Illeum	+	+++	+	+	+	++

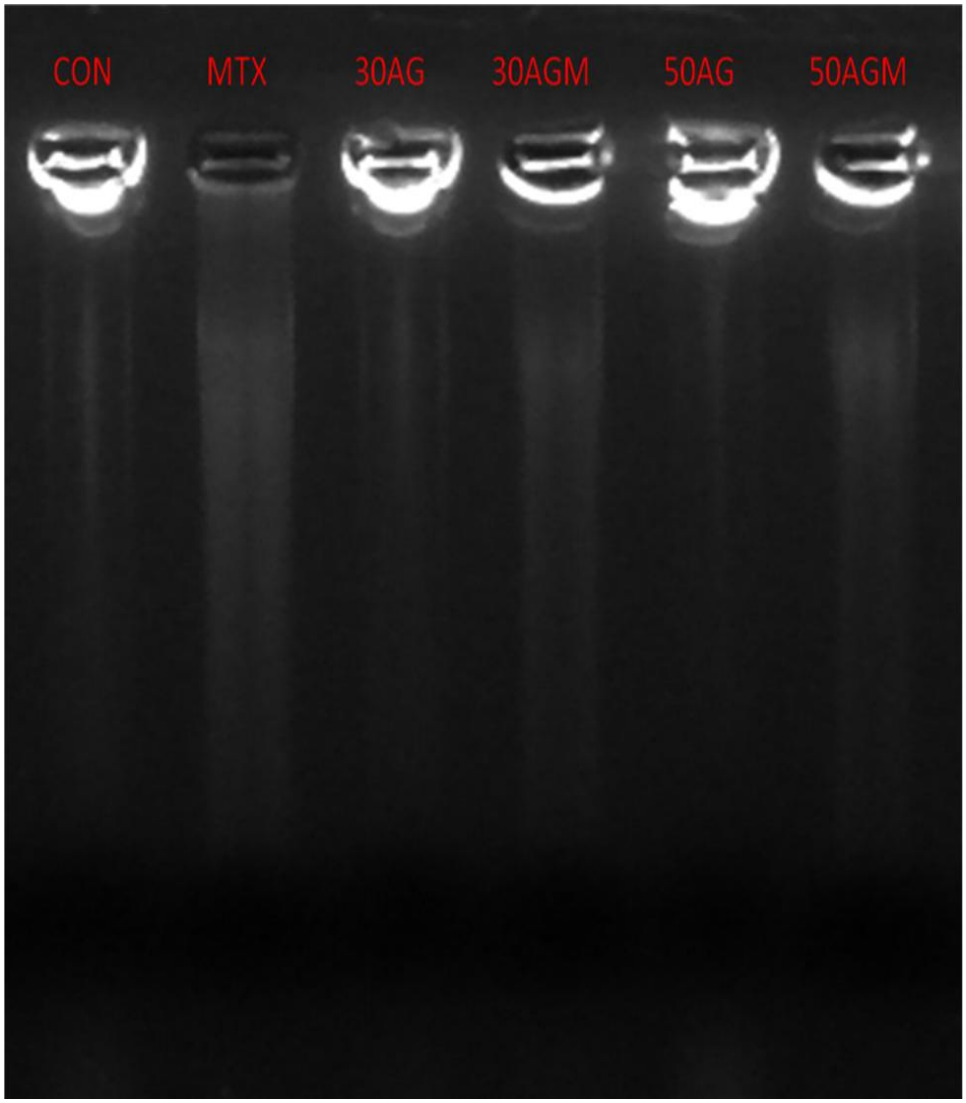
Figure: 6.5.34 PARP activation in nucleus by IF





Representative image of PARP on nucleus in the small intestine segments of rats 24 hours after the treatment with MTX and AG pretreated by immunofluorescence analysis (40X). MTX treated rats shown increased immunofluorescence of PARP on nucleus when compared to basal immunostaining in control rat small intestine tissue treated with vehicle. The expression was decreased in AG pretreated (30mg AGM and 50mg AGM) samples when compared to MTX.

Figure: 6.5.35 DNA FRAGMENTATION ASSAY



Effect of aminoguanidine on DNA fragmentation in methotrexate treated small intestine tissue. AG pretreatment protected against MTX induced DNA fragmentation.

6.5.6 DISCUSSION

We have examined in detail the role of NO in MTX induced intestinal mucositis. We have demonstrated induction of iNOS protein and mRNA expression, nitrate accumulation, increased protein tyrosine nitration, and selective nitration of proteins in the small intestines of MTX treated rats (chap 1). We have also shown that NFκB and its target inflammatory cytokines TNF and COX-2 contribute to MTX induced mucositis (chap 3). In addition, we have demonstrated (chap 4) that apoptotic pathway plays a role in MTX induced SI injury as evidenced by the release of cytochrome c from the mitochondria to the cytosol, activation of caspases 9 and 3, PARP cleavage and DNA fragmentation.

As MTX mucositis is associated with increased iNOS expressions and NO (PON) overproduction, we hypothesized that that the administration of AG, a selective iNOS inhibitor may protect against MTX induced SI injury by attenuating NO over production and PON formation. We first assessed the effects of AG pretreatment MTX induced morphological changes in the small intestines. MTX induced intestinal injury was assessed by histology using staining with hematoxylin and eosin. Cross-sections of small intestines from MTX treated rats demonstrated mucosa atrophy, broadening of the intestinal villi, degeneration and necrosis of epithelium cells .edema, and infiltration of inflammatory cells. When the animals were pretreated with AG, the development of these intestinal lesions was prevented in a dose-dependent manner. Thus, AG is protective against histological damage induced by MTX.

Biochemically, aminoguanidine pretreatment attenuated MTX induced iNOS protein expression and nitrite levels. Our findings are in agreement with those reported earlier (225). Other studies

have shown that aminoguanidine attenuates oral mucositis induced in rats by 5-FU treatment by blocking iNOS expression (239) and intestinal ulceration induced by indomethacin by suppressing NO production due to iNOS (448). Selective inhibition of iNOS by agents other than AG has been shown to protect against intestinal injury caused by different agents. Minocycline protects mice from gut injury induced by 5-FU by inhibiting iNOS (449), 400 W, a iNOS specific iNOS inhibitor s attenuates oral mucositis induced in rats by 5-FU treatment (239, 450). Anti-inflammatory agents such as CR3294 inhibit the expression of inducible nitric oxide synthase (iNOS) and protects the epithelium from injury in validated animal models of GI inflammation (439). Geldanamycin GA inhibits hemorrhage-induced iNOS overexpression, which diminishes the iNOS-induced cell injury (451). It is important to note that iNOS blockade has been shown to be beneficial in preventing I/R-induced gut injury/dysfunction (451-452). Inhibitory effect of baicalin on iNOS and NO expression in intestinal mucosa of rats with acute endotoxemia (254). These findings provide strong evidence for the role of iNOS in intestinal inflammation.

Nitration of protein tyrosine (3NT) is considered as foot print of PON formation. In this regard, we detected a significant increase of nitrotyrosine immunostaining in the intestinal segments of MTX-treated rats, reinforcing the role of NO via peroxynitrite on intestinal mucositis. Aminoguanidine pretreatment considerably reduced the immunostaining for nitrotyrosine suggesting that the decrease in the NT observed when aminoguanidine was administered to MTX-treated rats is probably related to suppression of peroxynitrite formation. Anti-inflammatory drugs like corticoids, indomethacin have been shown to be protective against peroxynitrite-mediated small intestinal injury and to decrease protein nitration levels (453-454).

NO and NF-κB signaling pathways are intimately linked. While, NF-κB activation is essential for NOS2 gene transcription, and NO regulates NF-κB at various points in its activation cascade. NO-related molecules modulate NF-κB signal transduction in a cell- and stimulus-specific manner, NO has been found to positively modulate NF-κB activation by affecting signaling cascades regulating inflammation (247). *In vivo* studies show that NO up-regulates NF-κB activation in situations like hemorrhagic shock and reperfusion injury (357). Therefore, we speculated that inhibition of iNOS may prevent NFκB activation. Indeed, in our study we found that AG pretreatment inhibits MTX induced NF-κB p65 nuclear translocation as evidenced by IF, IHC and WB. It has been demonstrated that interfering with iNOS ameliorates hemorrhagic shock- or sepsis-induced NF-κB activation in the gut. It has also been shown that iNOS protein and mRNA can be regulated transcriptionally via NFκB in the rat intestine (455). The resulting increased production of NO from iNOS may then produce intestinal injury and dysfunction directly or through the recruitment of leukocytes into the intestine. In several models of intestinal injury inhibition of NFκB has been shown to be beneficial in preventing intestinal injury, inflammation and lowering TNF, nitrite and iNOS expression (67).

We have demonstrated that MTX upregulates NF-κB inflammatory pathway resulting in the induction of its proinflammatory target gene such as, cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF)-α, iNOS, MMP 9, in the small intestines. Since AG pretreatment attenuated MTX induced activation of NFκB, we speculated that it may inhibit the production of the proinflammatory cytokines.

Therefore, we next assessed the effects of AG pretreatment on MTX induced changes in protein and mRNA expressions of COX-2, TNF, MMP2, and iNOS. AG pretreatment attenuated MTX included increased protein expressions of iNOS, COX-2, TNF but had no effect of their gene expression. These results suggest that the protective effect of AG is at the level of protein expression and not gene expression. In fact, ischemia/reperfusion injury in rat small intestine, aminoguanidine pretreatment protected against injury but had no effect on ischemia/reperfusion injury induced increased TNF mRNA (456).

As MTX induced small intestinal injury is associated with overproduction of NO by iNOS and increased enterocyte apoptosis as evidenced by cytc release, caspase 3 activation and DNA fragmentation, we speculated that iNOS inhibition and hence NO overproduction may prevent MTX induced apoptosis. AG pretreatment significantly prevented MTX induced cytochrome c release, caspase 3 activation, DNA damage, enterocyte apoptosis and intestinal injury. Two conclusions may be drawn from these findings 1. NO induced enhanced apoptosis plays role in MTX induced SI injury and 2. AG pretreatment protects SI from MTX induced SI injury by attenuating the apoptotic pathway. Studies have shown that many agents have protective effect against MTX induced intestinal mucositis by inhibiting enterocyte apoptosis (223, 457-464). However, in these studies apoptosis has been evaluated histologically and the components of the apoptotic pathway has not been studied in detail, to the best of our knowledge. Our studies and those reported earlier strongly support the role of apoptotic pathway in MTX induced intestinal damage.

Our results from chap 2 showed that MTX induced SI injury was associated with loss of activities of mitochondrial ETC complex including cytochrome oxidase (complex IV) and F1 ATPase and enzymes that are involved directly or indirectly in the citric acid cycle (e.g., aconitase, and succinate dehydrogenase),. Several studies have shown that these enzymes are targets for PON and that these enzymes undergo nitration and inactivation in inflammatory states. As MTX induced mucositis is associated with induction of iNOS and overproduction of NO, we speculated that loss of activities of these enzymes in the small intestines of rats after MTX treatment may be due to their nitration. Therefore, we hypothesized that aminoaguanidine pretreatment may prevent the loss of activities of these enzymes by attenuation of nitrosative stress. Indeed, AG pretreatment prevented MTX induced loss of activities of ETC complexes. The protective effect of AG on the ETC complexes may be due to its inhibition of iNOS, NO overproduction, and hence their nitration.

The direct antioxidant effect of AG may also contribute to the protective effect of AG against MTX induced SI injury as there is growing evidence for the role of aminoguanidine as an antioxidant. AG has been reported to be an effective hydroxyl radical scavenger (214). AG exhibits a significant dose-dependent effect against free radical damage. Aminoguanidine has been shown to exhibit trapping activity toward lipid-derived aldehydes such as MDA and 4-hydroxynonenal (215). Several studies have shown that AG acts as an antioxidant and can restore the antioxidants in the tissues as well as prevent cellular damage. AG treatment has been shown to alleviate the adverse effects of doxorubicin (DOX) in heart. by the reducing the levels of MDA, conjugated diene and restoration in the activities of antioxidant enzymes (465). Studies have shown that AG prevents gastric oxidative stress and nitric oxide formation as well as gastric

hemorrhagic erosion (466). Aminoguanidine has been shown to protect against the tissue damage associated with periodontitis by reducing nitric oxide production and oxidative stress, and increasing glutathione content (467). In a very recent study, aminoguanidine has been shown to reduce cisplatin induced nephrotoxicity due to its antioxidant effect (468). In another recent study, aminoguanidine has been shown to increase the endogenous antioxidant defense mechanism in rats and protect the animals from radiation-induced lung toxicity (469). Abdel-Zaher et al.(470) have shown that AG markedly inhibits acetaminophen -induced hepatic and renal depletion of antioxidants as well as nitric oxide overproduction.

Therefore, we speculated that AG antioxidant property may also contribute to its protective effect against MTX induced mucositis, in addition to its action as an iNOS inhibitor. Our studies show that AG pretreatment partially but significantly restored MTX induced decreased activities of the antioxidant enzymes, namely, catalase, GST, SOD, and carbonic anhydrase. These findings suggest that the antioxidant property of AG may also contribute to its protective effect against MTX induced mucositis.

In conclusion, AG appears to attenuate the severity of small-intestine mucositis induced in rats by MTX treatment. The therapeutic mechanisms of AG may involve inhibition of inducible nitric oxide synthase (iNOS), suppression of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) COX-2 and inhibition of matrix metalloproteinase expression, cytochrome c release, caspase activation, and (PARP-1) cleavage. The antioxidant property of AG may also contribute to its protective effect on MTX enteritis.

CHAPTER – 7

Summary and Conclusion

SUMMARY AND CONCLUSION OF THE STUDIES

The ultimate purpose of the study was to identify the modifications and consequence due to nitrosative stress by which MTX causes small intestinal damage. Increased nitric oxide production was found to be associated with gut barrier failure and inflammation. In the first study, we have assessed the role the nitrosative stress in MTX induced small intestinal damage by measuring the nitrosative stress parameters in the small intestine samples at 24 hours after 3 consecutive intraperitoneal injections of MTX at the dose of 7 mg / kg body weight. Nitric oxide level (Nitrite + Nitrate), expression of inducible nitric oxide synthase and nitrotyrosine was increased in MTX treated samples.

We also assessed peroxynitrite induced protein tyrosine nitration, subcellular distribution on nitrated proteins to identify the proteins that may undergo nitration. We found many proteins either increase or decrease expression of nitrotyrosine modification in the cell fractions including homogenate, nucleus, mitochondria, microsomes and cytosol. Thus, it is concluded that there is strong association of nitrosative stress that lead to nitration of proteins to the extent of major subcellular fractions may play a role in MTX induced mucositis.

First study suggested the role of nitrotyrosine but not consequence of nitration in MTX induced small intestine damage. Nitrosative stress leads to modification in the activity and function of enzymes that are protein in nature. Therefore, the second study was designed to determine the alteration in the activity of enzymes thay may undergo nitration. Therefore, we assessed the activity of enzymes those we speculate proteins from the first study mainly ETC complexes, TCA cycle and antioxidant enzymes. The ETC complex enzymes - cytochrome-c-oxidase and

F1-ATPase enzyme activity was significantly decreased in MTX treated samples when compared to control. The TCA cycle enzymes - aconitase, succinate deydrogenase, creatine kinase and glutamate dehydrogenase were significantly decreased in MTX treated samples when compared to control. The antioxidant enzymes - catalase, glutathione peroxidase, glutathione-s-transferase, carbonic anhydrase enzyme activity was significantly decreased and superoxide was increased in MTX treated samples as compared to control. This result indicates that MTX alters the activity of the enzymes that are known to undergo nitration and inactivation. MTX induced loss of ETC complex activity and depletion of antioxidant enzymes may contribute to mitochondrial injury, enterocyte apoptosis, and small intestinal injury.

Previous studies have shown that tyrosine nitration triggers dissociation of IκB from NuclearFactor-κB (NF-κB), which results in activation of this pathway that plays an important role in cancer and inflammation. Therefore, the third study was designed to assess the role of inflammatory signaling pathway in MTX induced small intestine damage. MTX treatment resulted in NFκB activation and nuclear translocation as evidenced by immunofluorescence, immunohistochemitry and immunoblot analysis of intestinal segments. We also found that there is significant increased expression of NFκB target proteins and genes including TNFα, COX2, iNOS, PLA_2, MMP2, MMP9, HO1 and HSP70 in MTX treated samples as compared to control. These results concluded that there is activation of NFκB signaling inflammatory pathway in MTX treated small intestine samples.

Studies have been shown that nitration of cytochrome-c leads to the release from mitochondria to cytosol and activates caspase cascade leading to activation of apoptosis pathway. Therefore, the

fourth study was designed to assess the role of apoptotic signaling pathway in MTX induced small intestine damage. The expression of CYT-C was decreased in mitochondria and increased in cytosol fraction in MTX treated samples as compared to control. We also found significant increase in active caspases expression (CASP-9 and CASP-3), PARP expression and DNA fragmentation in the MTX treated small intestine samples as compared to control. Thus, it is concluded that there is activation of intrinsic apoptotic signaling pathway in MTX treated small intestine samples.

Aminoguanidine, specific inhibitor of iNOS reduces the nitrosative stress induced damages. Thus, the last study was designed to investigate the protective effect of aminoguanidine in MTX induced small intestine damage. Rats were pretreated with two different concentrations (30mg and 50mg per kg body weight) of aminoguanidine one hour prior to MTX administration. There is improvement in the morphology of the damaged small intestine section in aminoguanidine pretreated group when compared to MTX treated group. Thus, aminoguanidine attenuated MTX induced small intestine damage by light microscopy. The nitrosative stress parameters like nitric oxide levels, expression of iNOS and NTY was decreased and the alteration in the activity of enzymes in MTX treated samples was reversed by aminoguanidine pretreatment. Thus, aminoguanidine abolished the increased nitrosative stress in response to MTX.

AG pretreatment attenuated MTX induced upregulation of NFκB and its proinflammatory target genes protein expressions, iNOS, TNF, COX-2 and PLA_2. AG pretreatment attenuated MTX induced mitochondrial apoptotic pathway, CYT-C, CASP-9, CASP-3, PARP cleavage, and DNA fragmentation. However, AG pretreatment had no significant effect on the mRNA expressions of

these proteins. In conclusion, AG pretreatment improved MTX induced morphological changes in the small intestine inhibited the NFκB inflammatory pathway and apoptotic pathway. Thus, aminoguanidine has a protective role in MTX induced mucositis.

Aminoguanidine has been administered in both physiological and pharmacological amounts to humans and animals, and there is widespread agreement that it is non-toxic even at higher concentration up to 500mg. It is therefore concluded that aminoguanidine appeared to be potential agent that reduce MTX induced small intestinal damage.

CHAPTER – 8

Future plans

The results of the current studies reveal the role of nitrosative stress, modification of proteins and enzymes due to nitrosative stress, activation of inflammatory and apoptosis signaling pathway in MTX induced small intestine damage of rat. Further studies are required to check the specific list of proteins that undergo nitration after methotrexate therapy.

Aminoguanidine used in the studies has been shown to protect against MTX induced small intestinal damage in a rat model. Thus, aminoguanidine shows potential extend to be used in humans. The possible benefits of aminoguanidine need to be explored further and its clinical utility need to be assessed in patients to ascertain whether they can be recommended for use in pathiets who need to take methotrexate therapy on a long-term basis.

In future, we plan to carry out the following studies

1. Identify the individual proteins that are nitrated in the small intestines of rats after MTX treatment, using mass spectrometry
2. To study the role of protein cysteine nitrosylation in MTX induced enteritis
3. To investigate whether the administration of NFkb inhibitors can attenuate MTX enteritis
4. To measure the levels of nitrites, iNOS protein and protein tyrosine adducts in the serum of patients on MTX therapy

CHAPTER – 9

Bibliography

1. Sonis ST, Lindquist L, Van Vugt A, Stewart AA, Stam K, Qu GY, et al. Prevention of chemotherapy-induced ulcerative mucositis by transforming growth factor beta 3. Cancer Res. 1994;54(5):1135-8.

2. Naidu MU, Ramana GV, Rani PU, Mohan IK, Suman A, Roy P. Chemotherapy-induced and/or radiation therapy-induced oral mucositis--complicating the treatment of cancer. Neoplasia. 2004;6(5):423-31.

3. Sonis ST, Elting LS, Keefe D, Peterson DE, Schubert M, Hauer-Jensen M, et al. Perspectives on cancer therapy-induced mucosal injury: pathogenesis, measurement, epidemiology, and consequences for patients. Cancer. 2004;100(9 Suppl):1995-2025.

4. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. N Engl J Med. 1983;309(18):1094-104.

5. Wilke WS. Methotrexate use in miscellaneous inflammatory diseases. Rheum Dis Clin North Am. 1997;23(4):855-82.

6. Hashkes PJ, Becker ML, Cabral DA, Laxer RM, Paller AS, Rabinovich CE, et al. Methotrexate: new uses for an old drug. J Pediatr. 2014;164(2):231-6.

7. Saag KG, Teng GG, Patkar NM, Anuntiyo J, Finney C, Curtis JR, et al. American College of Rheumatology 2008 recommendations for the use of nonbiologic and biologic disease-modifying antirheumatic drugs in rheumatoid arthritis. Arthritis Rheum. 2008;59(6):762-84.

8. Smolen JS, Landewe R, Breedveld FC, Buch M, Burmester G, Dougados M, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update. Ann Rheum Dis. 2014;73(3):492-509.

9. Gossec L, Smolen JS, Gaujoux-Viala C, Ash Z, Marzo-Ortega H, van der Heijde D, et al. European League Against Rheumatism recommendations for the management of psoriatic arthritis with pharmacological therapies. Ann Rheum Dis. 2012;71(1):4-12.

10. Gottlieb A, Korman NJ, Gordon KB, Feldman SR, Lebwohl M, Koo JY, et al. Guidelines of care for the management of psoriasis and psoriatic arthritis: Section 2. Psoriatic arthritis: overview and guidelines of care for treatment with an emphasis on the biologics. J Am Acad Dermatol. 2008;58(5):851-64.

11. Dignass A, Van Assche G, Lindsay JO, Lemann M, Soderholm J, Colombel JF, et al. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Current management. J Crohns Colitis. 2010;4(1):28-62.

12. Conway R, Low C, Coughlan RJ, O'Donnell MJ, Carey JJ. Methotrexate use and risk of lung disease in psoriasis, psoriatic arthritis, and inflammatory bowel disease: systematic

literature review and meta-analysis of randomised controlled trials. BMJ. 2015;350:h1269.

13. Al-Quteimat OM, Al-Badaineh MA. Practical issues with high dose methotrexate therapy. Saudi Pharm J. 2014;22(4):385-7.

14. Copur S, Dahut W, Chu E, Allegra CJ. Bone marrow aplasia and severe skin rash after a single low dose of methotrexate. Anticancer Drugs. 1995;6(1):154-7.

15. Wolfgang WJ. Exploring protection from methotrexate-induced teratogenicity in flies. Toxicol Sci. 2007;99(2):363-5.

16. Neves C, Jorge R, Barcelos A. [The network of methotrexate toxicity]. Acta Reumatol Port. 2009;34(1):11-34. A teia de toxicidade do metotrexato.

17. Bleyer WA. Methotrexate: clinical pharmacology, current status and therapeutic guidelines. Cancer Treat Rev. 1977;4(2):87-101.

18. Hoekstra M, van Ede AE, Haagsma CJ, van de Laar MA, Huizinga TW, Kruijsen MW, et al. Factors associated with toxicity, final dose, and efficacy of methotrexate in patients with rheumatoid arthritis. Ann Rheum Dis. 2003;62(5):423-6.

19. Pinkerton CR, Booth IW, Milla PJ. Topical methotrexate alters solute and water transport in the rat jejunum in vivo and rabbit ileum in vitro. Gut. 1985;26(7):704-9.

20. Pinkerton CR, Cameron CH, Sloan JM, Glasgow JF, Gwevava NJ. Jejunal crypt cell abnormalities associated with methotrexate treatment in children with acute lymphoblastic leukaemia. J Clin Pathol. 1982;35(11):1272-7.

21. Taminiau JA, Gall DG, Hamilton JR. Response of the rat small-intestine epithelium to methotrexate. Gut. 1980;21(6):486-92.

22. de Thurah A, Norgaard M, Johansen MB, Stengaard-Pedersen K. Methotrexate compliance among patients with rheumatoid arthritis: the influence of disease activity, disease duration, and co-morbidity in a 10-year longitudinal study. Scand J Rheumatol. 2010;39(3):197-205.

23. Kolli V, Natarajan K, Isaac B, Selvakumar D, Abraham P. Mitochondrial dysfunction and respiratory chain defects in a rodent model of methotrexate-induced enteritis. Hum Exp Toxicol. 2013.

24. Kolli VK, Abraham P, Isaac B, Selvakumar D. Neutrophil infiltration and oxidative stress may play a critical role in methotrexate-induced renal damage. Chemotherapy. 2009;55(2):83-90.

25. Kolli VK, Abraham P, Rabi S. Methotrexate-induced nitrosative stress may play a critical role in small intestinal damage in the rat. Arch Toxicol. 2008;82(10):763-70.

26. Liu FS. Mechanisms of chemotherapeutic drug resistance in cancer therapy--a quick review. Taiwan J Obstet Gynecol. 2009;48(3):239-44.

27. Maccormick RE. Possible acceleration of aging by adjuvant chemotherapy: a cause of early onset frailty? Med Hypotheses. 2006;67(2):212-5.

28. Bertino JR. Cancer research: from folate antagonism to molecular targets. Best Pract Res Clin Haematol. 2009;22(4):577-82.

29. Sagar J, Chaib B, Sales K, Winslet M, Seifalian A. Role of stem cells in cancer therapy and cancer stem cells: a review. Cancer Cell Int. 2007;7:9.

30. Wu XZ. A new classification system of anticancer drugs - based on cell biological mechanisms. Med Hypotheses. 2006;66(5):883-7.

31. Berruti A, Brizzi MP, Generali D, Ardine M, Dogliotti L, Bruzzi P, et al. Presurgical systemic treatment of nonmetastatic breast cancer: facts and open questions. Oncologist. 2008;13(11):1137-48.

32. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. Cell. 2002;108(2):153-64.

33. Nichols JW, Bae YH. Odyssey of a cancer nanoparticle: from injection site to site of action. Nano Today. 2012;7(6):606-18.

34. Ma H, Das T, Pereira S, Yang Z, Zhao M, Mukerji P, et al. Efficacy of dietary antioxidants combined with a chemotherapeutic agent on human colon cancer progression in a fluorescent orthotopic mouse model. Anticancer Res. 2009;29(7):2421-6.

35. Al-Tonbary Y, Al-Haggar M, El-Ashry R, El-Dakroory S, Azzam H, Fouda A. Vitamin e and N-acetylcysteine as antioxidant adjuvant therapy in children with acute lymphoblastic leukemia. Adv Hematol. 2009;2009:689639.

36. Krajinovic M, Moghrabi A. Pharmacogenetics of methotrexate. Pharmacogenomics. 2004;5(7):819-34.

37. Liegler DG, Henderson ES, Hahn MA, Oliverio VT. The effect of organic acids on renal clearance of methotrexate in man. Clin Pharmacol Ther. 1969;10(6):849-57.

38. Choi HK, Hernan MA, Seeger JD, Robins JM, Wolfe F. Methotrexate and mortality in patients with rheumatoid arthritis: a prospective study. Lancet. 2002;359(9313):1173-7.

39. Wasko MC, Dasgupta A, Hubert H, Fries JF, Ward MM. Propensity-adjusted association of methotrexate with overall survival in rheumatoid arthritis. Arthritis Rheum. 2013;65(2):334-42.

40. Goodsell DS. The molecular perspective: methotrexate. Oncologist. 1999;4(4):340-1.

41. Rajagopalan PT, Zhang Z, McCourt L, Dwyer M, Benkovic SJ, Hammes GG. Interaction of dihydrofolate reductase with methotrexate: ensemble and single-molecule kinetics. Proc Natl Acad Sci U S A. 2002;99(21):13481-6.

42. Cronstein BN. The mechanism of action of methotrexate. Rheum Dis Clin North Am. 1997;23(4):739-55.

43. Wessels JA, Huizinga TW, Guchelaar HJ. Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis. Rheumatology (Oxford). 2008;47(3):249-55.

44. Hamilton RA, Kremer JM. The effects of food on methotrexate absorption. J Rheumatol. 1995;22(4):630-2.

45. Hoekstra M, Haagsma C, Neef C, Proost J, Knuif A, van de Laar M. Bioavailability of higher dose methotrexate comparing oral and subcutaneous administration in patients with rheumatoid arthritis. J Rheumatol. 2004;31(4):645-8.

46. Braun J. Optimal administration and dosage of methotrexate. Clin Exp Rheumatol. 2010;28(5 Suppl 61):S46-51.

47. Kristensen LO, Weismann K, Hutters L. Renal function and the rate of disappearance of methotrexate from serum. Eur J Clin Pharmacol. 1975;8(6):439-44.

48. Soon SY, Ansari A, Yaneza M, Raoof S, Hirst J, Sanderson JD. Experience with the use of low-dose methotrexate for inflammatory bowel disease. Eur J Gastroenterol Hepatol. 2004;16(9):921-6.

49. van der Meer A, Wulffraat NM, Prakken BJ, Gijsbers B, Rademaker CM, Sinnema G. Psychological side effects of MTX treatment in juvenile idiopathic arthritis: a pilot study. Clin Exp Rheumatol. 2007;25(3):480-5.

50. Lu Z, Ding L, Lu Q, Chen YH. Claudins in intestines: Distribution and functional significance in health and diseases. Tissue Barriers. 2013;1(3):e24978.

51. Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. Clin Exp Immunol. 2007;147(2):227-35.

52. Coruzzi G. Overview of gastrointestinal toxicology. Curr Protoc Toxicol. 2010;Chapter 21:Unit 21 1.

53. Kim YJ, Kim EH, Hahm KB. Oxidative stress in inflammation-based gastrointestinal tract diseases: challenges and opportunities. J Gastroenterol Hepatol. 2012;27(6):1004-10.

54. Martin GR, Wallace JL. Gastrointestinal inflammation: a central component of mucosal defense and repair. Exp Biol Med (Maywood). 2006;231(2):130-7.

55. Wallace JL, Chin BC. Inflammatory mediators in gastrointestinal defense and injury. Proc Soc Exp Biol Med. 1997;214(3):192-203.

56. Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. Cytokine Growth Factor Rev. 2002;13(4-5):413-21.

57. Wallace JL, Granger DN. The cellular and molecular basis of gastric mucosal defense. FASEB J. 1996;10(7):731-40.

58. Keefe DM, Brealey J, Goland GJ, Cummins AG. Chemotherapy for cancer causes apoptosis that precedes hypoplasia in crypts of the small intestine in humans. Gut. 2000;47(5):632-7.

59. Stringer AM, Gibson RJ, Bowen JM, Logan RM, Yeoh AS, Keefe DM. Chemotherapy-induced mucositis: the role of gastrointestinal microflora and mucins in the luminal environment. J Support Oncol. 2007;5(6):259-67.

60. Logan RM, Stringer AM, Bowen JM, Yeoh AS, Gibson RJ, Sonis ST, et al. The role of pro-inflammatory cytokines in cancer treatment-induced alimentary tract mucositis: pathobiology, animal models and cytotoxic drugs. Cancer Treat Rev. 2007;33(5):448-60.

61. Silverman S, Jr. Diagnosis and management of oral mucositis. J Support Oncol. 2007;5(2 Suppl 1):13-21.

62. Gibson RJ, Bowen JM, Alvarez E, Finnie J, Keefe DM. Establishment of a single-dose irinotecan model of gastrointestinal mucositis. Chemotherapy. 2007;53(5):360-9.

63. Keefe DM, Gibson RJ, Hauer-Jensen M. Gastrointestinal mucositis. Semin Oncol Nurs. 2004;20(1):38-47.

64. Gibson RJ, Bowen JM, Cummins AG, Keefe DM. Relationship between dose of methotrexate, apoptosis, p53/p21 expression and intestinal crypt proliferation in the rat. Clin Exp Med. 2005;4(4):188-95.

65. Sonis ST. Mucositis as a biological process: a new hypothesis for the development of chemotherapy-induced stomatotoxicity. Oral Oncol. 1998;34(1):39-43.

66. Bowen JM, Gibson RJ, Cummins AG, Keefe DM. Intestinal mucositis: the role of the Bcl-2 family, p53 and caspases in chemotherapy-induced damage. Support Care Cancer. 2006;14(7):713-31.

67. Sonis ST. The biologic role for nuclear factor-kappaB in disease and its potential involvement in mucosal injury associated with anti-neoplastic therapy. Crit Rev Oral Biol Med. 2002;13(5):380-9.

68. Hall PD, Benko H, Hogan KR, Stuart RK. The influence of serum tumor necrosis factor-alpha and interleukin-6 concentrations on nonhematologic toxicity and hematologic recovery in patients with acute myelogenous leukemia. Exp Hematol. 1995;23(12):1256-60.

69. Sonis ST. The pathobiology of mucositis. Nat Rev Cancer. 2004;4(4):277-84.

70. Sonis ST, O'Donnell KE, Popat R, Bragdon C, Phelan S, Cocks D, et al. The relationship between mucosal cyclooxygenase-2 (COX-2) expression and experimental radiation-induced mucositis. Oral Oncol. 2004;40(2):170-6.

71. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39(1):44-84.

72. Rios-Arrabal S, Artacho-Cordon F, Leon J, Roman-Marinetto E, Del Mar Salinas-Asensio M, Calvente I, et al. Involvement of free radicals in breast cancer. Springerplus. 2013;2:404.

73. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol. 2000;279(6):L1005-28.

74. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003;552(Pt 2):335-44.

75. Bus JS, Gibson JE. Mechanisms of superoxide radical-mediated toxicity. J Toxicol Clin Toxicol. 1982;19(6-7):689-97.

76. Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease. Clin Chem. 2006;52(4):601-23.

77. Patel KK, Stappenbeck TS. Autophagy and intestinal homeostasis. Annu Rev Physiol. 2013;75:241-62.

78. Pasparakis M. Role of NF-kappaB in epithelial biology. Immunol Rev. 2012;246(1):346-58.

79. Biasi F, Leonarduzzi G, Oteiza PI, Poli G. Inflammatory bowel disease: mechanisms, redox considerations, and therapeutic targets. Antioxid Redox Signal. 2013;19(14):1711-47.

80. Circu ML, Aw TY. Intestinal redox biology and oxidative stress. Semin Cell Dev Biol. 2012;23(7):729-37.

81. Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. Oxidative Stress: An Essential Factor in the Pathogenesis of Gastrointestinal Mucosal Diseases. Physiological reviews. 2014;94(2):329-54.

82. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev. 1991;43(2):109-42.

83. Geller DA, Billiar TR. Molecular biology of nitric oxide synthases. Cancer Metastasis Rev. 1998;17(1):7-23.

84. Davis KL, Martin E, Turko IV, Murad F. Novel effects of nitric oxide. Annu Rev Pharmacol Toxicol. 2001;41:203-36.

85. Zweier JL, Wang P, Samouilov A, Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues. Nat Med. 1995;1(8):804-9.

86. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A. 1990;87(4):1620-4.

87. Patel RP, McAndrew J, Sellak H, White CR, Jo H, Freeman BA, et al. Biological aspects of reactive nitrogen species. Biochim Biophys Acta. 1999;1411(2-3):385-400.

88. Wang Y, Marsden PA. Nitric oxide synthases: biochemical and molecular regulation. Curr Opin Nephrol Hypertens. 1995;4(1):12-22.

89. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J. 1994;298 (Pt 2):249-58.

90. Guix FX, Uribesalgo I, Coma M, Munoz FJ. The physiology and pathophysiology of nitric oxide in the brain. Prog Neurobiol. 2005;76(2):126-52.

91. Forstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J. 2012;33(7):829-37, 37a-37d.

92. Huang PL. Neuronal and endothelial nitric oxide synthase gene knockout mice. Braz J Med Biol Res. 1999;32(11):1353-9.

93. Rao KM. Molecular mechanisms regulating iNOS expression in various cell types. J Toxicol Environ Health B Crit Rev. 2000;3(1):27-58.

94. Blanchette J, Jaramillo M, Olivier M. Signalling events involved in interferon-gamma-inducible macrophage nitric oxide generation. Immunology. 2003;108(4):513-22.

95. Kroncke KD, Suschek CV, Kolb-Bachofen V. Implications of inducible nitric oxide synthase expression and enzyme activity. Antioxid Redox Signal. 2000;2(3):585-605.

96. Missall TA, Lodge JK, McEwen JE. Mechanisms of resistance to oxidative and nitrosative stress: implications for fungal survival in mammalian hosts. Eukaryot Cell. 2004;3(4):835-46.

97. Gow AJ, Farkouh CR, Munson DA, Posencheg MA, Ischiropoulos H. Biological significance of nitric oxide-mediated protein modifications. Am J Physiol Lung Cell Mol Physiol. 2004;287(2):L262-8.

98. Ischiropoulos H, al-Mehdi AB. Peroxynitrite-mediated oxidative protein modifications. FEBS Lett. 1995;364(3):279-82.

99. Wallace JL, Miller MJ. Nitric oxide in mucosal defense: a little goes a long way. Gastroenterology. 2000;119(2):512-20.

100. Marcinkiewicz J, Chain B, Nowak B, Grabowska A, Bryniarski K, Baran J. Antimicrobial and cytotoxic activity of hypochlorous acid: interactions with taurine and nitrite. Inflamm Res. 2000;49(6):280-9.

101. Sanders KM, Ward SM. Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. Am J Physiol. 1992;262(3 Pt 1):G379-92.

102. Alican I, Kubes P. A critical role for nitric oxide in intestinal barrier function and dysfunction. Am J Physiol. 1996;270(2 Pt 1):G225-37.

103. Martin MJ, Jimenez MD, Motilva V. New issues about nitric oxide and its effects on the gastrointestinal tract. Curr Pharm Des. 2001;7(10):881-908.

104. Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82(1):47-95.

105. Hancock JT, Desikan R, Neill SJ. Role of reactive oxygen species in cell signalling pathways. Biochem Soc Trans. 2001;29(Pt 2):345-50.

106. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87(1):315-424.

107. Ischiropoulos H. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch Biochem Biophys. 1998;356(1):1-11.

108. Radi R, Cassina A, Hodara R, Quijano C, Castro L. Peroxynitrite reactions and formation in mitochondria. Free Radic Biol Med. 2002;33(11):1451-64.

109. Szabo C, Ischiropoulos H, Radi R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nat Rev Drug Discov. 2007;6(8):662-80.

110. Souza JM, Daikhin E, Yudkoff M, Raman CS, Ischiropoulos H. Factors determining the selectivity of protein tyrosine nitration. Arch Biochem Biophys. 1999;371(2):169-78.

111. Abello N, Kerstjens HA, Postma DS, Bischoff R. Protein tyrosine nitration: selectivity, physicochemical and biological consequences, denitration, and proteomics methods for the identification of tyrosine-nitrated proteins. J Proteome Res. 2009;8(7):3222-38.

112. Jia M, Mateoiu C, Souchelnytskyi S. Protein tyrosine nitration in the cell cycle. Biochem Biophys Res Commun. 2011;413(2):270-6.

113. Radi R. Protein tyrosine nitration: biochemical mechanisms and structural basis of functional effects. Acc Chem Res. 2013;46(2):550-9.

114. Szabo C. Multiple pathways of peroxynitrite cytotoxicity. Toxicol Lett. 2003;140-141:105-12.

115. Cassina AM, Hodara R, Souza JM, Thomson L, Castro L, Ischiropoulos H, et al. Cytochrome c nitration by peroxynitrite. J Biol Chem. 2000;275(28):21409-15.

116. Ji Y, Bennett BM. Activation of microsomal glutathione s-transferase by peroxynitrite. Mol Pharmacol. 2003;63(1):136-46.

117. Yeo WS, Lee SJ, Lee JR, Kim KP. Nitrosative protein tyrosine modifications: biochemistry and functional significance. BMB Rep. 2008;41(3):194-203.

118. Banan A, Fields JZ, Decker H, Zhang Y, Keshavarzian A. Nitric oxide and its metabolites mediate ethanol-induced microtubule disruption and intestinal barrier dysfunction. J Pharmacol Exp Ther. 2000;294(3):997-1008.

119. Potoka DA, Upperman JS, Zhang XR, Kaplan JR, Corey SJ, Grishin A, et al. Peroxynitrite inhibits enterocyte proliferation and modulates Src kinase activity in vitro. Am J Physiol Gastrointest Liver Physiol. 2003;285(5):G861-9.

120. Hess DT, Stamler JS. Regulation by S-nitrosylation of protein post-translational modification. J Biol Chem. 2012;287(7):4411-8.

121. Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, et al. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. Science. 2002;297(5584):1186-90.

122. Abramson SB, Amin AR, Clancy RM, Attur M. The role of nitric oxide in tissue destruction. Best Pract Res Clin Rheumatol. 2001;15(5):831-45.

123. Sharma JN, Al-Omran A, Parvathy SS. Role of nitric oxide in inflammatory diseases. Inflammopharmacology. 2007;15(6):252-9.

124. Beinke S, Ley SC. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. Biochem J. 2004;382(Pt 2):393-409.

125. Guzik TJ, Korbut R, Adamek-Guzik T. Nitric oxide and superoxide in inflammation and immune regulation. J Physiol Pharmacol. 2003;54(4):469-87.

126. Taylor HR, Semba RD, Newland HS, Keyvan-Larijani E, White A, Dukuly Z, et al. Ivermectin treatment of patients with severe ocular onchocerciasis. Am J Trop Med Hyg. 1989;40(5):494-500.

127. Baldwin AS, Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol. 1996;14:649-83.

128. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene. 1999;18(49):6853-66.

129. Heller RA, Kronke M. Tumor necrosis factor receptor-mediated signaling pathways. J Cell Biol. 1994;126(1):5-9.

130. Li Q, Verma IM. NF-kappaB regulation in the immune system. Nat Rev Immunol. 2002;2(10):725-34. 131. Hanazawa T, Kharitonov SA, Barnes PJ. Increased nitrotyrosine in exhaled breath condensate of patients with asthma. Am J Respir Crit Care Med. 2000;162(4 Pt 1):1273-6.

132. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol. 2009;1(6):a001651.

133. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med. 1997;336(15):1066-71.

134. Spehlmann ME, Eckmann L. Nuclear factor-kappa B in intestinal protection and destruction. Curr Opin Gastroenterol. 2009;25(2):92-9.

135. Karrasch T, Jobin C. NF-kappaB and the intestine: friend or foe? Inflamm Bowel Dis. 2008;14(1):114-24.

136. Walsh LJ, Trinchieri G, Waldorf HA, Whitaker D, Murphy GF. Human dermal mast cells contain and release tumor necrosis factor alpha, which induces endothelial leukocyte adhesion molecule 1. Proc Natl Acad Sci U S A. 1991;88(10):4220-4.

137. Camussi G, Albano E, Tetta C, Bussolino F. The molecular action of tumor necrosis factor-alpha. Eur J Biochem. 1991;202(1):3-14.

138. Ware CF, VanArsdale S, VanArsdale TL. Apoptosis mediated by the TNF-related cytokine and receptor families. J Cell Biochem. 1996;60(1):47-55.

139. Warzocha K, Bienvenu J, Coiffier B, Salles G. Mechanisms of action of the tumor necrosis factor and lymphotoxin ligand-receptor system. Eur Cytokine Netw. 1995;6(2):83-96.

140. Idriss HT, Naismith JH. TNF alpha and the TNF receptor superfamily: structure-function relationship(s). Microsc Res Tech. 2000;50(3):184-95.

141. Wild GE, Waschke KA, Bitton A, Thomson AB. The mechanisms of prednisone inhibition of inflammation in Crohn's disease involve changes in intestinal permeability, mucosal TNFalpha production and nuclear factor kappa B expression. Aliment Pharmacol Ther. 2003;18(3):309-17.

142. Bertrand V, Guimbaud R, Tulliez M, Mauprivez C, Sogni P, Couturier D, et al. Increase in tumor necrosis factor-alpha production linked to the toxicity of indomethacin for the rat small intestine. Br J Pharmacol. 1998;124(7):1385-94.

143. Hang CH, Shi JX, Li JS, Li WQ, Yin HX. Up-regulation of intestinal nuclear factor kappa B and intercellular adhesion molecule-1 following traumatic brain injury in rats. World J Gastroenterol. 2005;11(8):1149-54.

144. Ong ZY, Gibson RJ, Bowen JM, Stringer AM, Darby JM, Logan RM, et al. Pro-inflammatory cytokines play a key role in the development of radiotherapy-induced gastrointestinal mucositis. Radiat Oncol. 2010;5:22.

145. Sonis ST. Regimen-related gastrointestinal toxicities in cancer patients. Curr Opin Support Palliat Care. 2010;4(1):26-30.

146. Botting RM. Inhibitors of cyclooxygenases: mechanisms, selectivity and uses. J Physiol Pharmacol. 2006;57 Suppl 5:113-24.

147. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, et al. Cyclooxygenase in biology and disease. FASEB J. 1998;12(12):1063-73.

148. Xu L, Han C, Lim K, Wu T. Activation of cytosolic phospholipase A2alpha through nitric oxide-induced S-nitrosylation. Involvement of inducible nitric-oxide synthase and cyclooxygenase-2. J Biol Chem. 2008;283(6):3077-87.

149. Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol. 1998;38:97-120.

150. Sklyarov AY, Panasyuk NB, Fomenko IS. Role of nitric oxide-synthase and cyclooxygenase/lipooxygenase systems in development of experimental ulcerative colitis. J Physiol Pharmacol. 2011;62(1):65-73.

151. Wallace JL, Bak A, McKnight W, Asfaha S, Sharkey KA, MacNaughton WK. Cyclooxygenase 1 contributes to inflammatory responses in rats and mice: implications for gastrointestinal toxicity. Gastroenterology. 1998;115(1):101-9.

152. Grishin AV, Wang J, Potoka DA, Hackam DJ, Upperman JS, Boyle P, et al. Lipopolysaccharide induces cyclooxygenase-2 in intestinal epithelium via a noncanonical p38 MAPK pathway. J Immunol. 2006;176(1):580-8.

153. Hoshino T, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T, Mizushima T. Prostaglandin E2 protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation. J Biol Chem. 2003;278(15):12752-8.

154. Joseph RR, Yazer E, Hanakawa Y, Stadnyk AW. Prostaglandins and activation of AC/cAMP prevents anoikis in IEC-18. Apoptosis. 2005;10(6):1221-33.

155. Lugo B, Ford HR, Grishin A. Molecular signaling in necrotizing enterocolitis: regulation of intestinal COX-2 expression. J Pediatr Surg. 2007;42(7):1165-71.

156. Chung DH, Ethridge RT, Kim S, Owens-Stovall S, Hernandez A, Kelly DR, et al. Molecular mechanisms contributing to necrotizing enterocolitis. Ann Surg. 2001;233(6):835-42.

157. Sziksz E, Pap D, Veres G, Fekete A, Tulassay T, Vannay A. Involvement of heat shock proteins in gluten-sensitive enteropathy. World J Gastroenterol. 2014;20(21):6495-503.

158. Pratt WB, Galigniana MD, Morishima Y, Murphy PJ. Role of molecular chaperones in steroid receptor action. Essays Biochem. 2004;40:41-58.

159. Osterloh A, Breloer M. Heat shock proteins: linking danger and pathogen recognition. Med Microbiol Immunol. 2008;197(1):1-8.

160. Dudeja V, Mujumdar N, Phillips P, Chugh R, Borja-Cacho D, Dawra RK, et al. Heat shock protein 70 inhibits apoptosis in cancer cells through simultaneous and independent mechanisms. Gastroenterology. 2009;136(5):1772-82.

161. Kim YM, de Vera ME, Watkins SC, Billiar TR. Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-alpha-induced apoptosis by inducing heat shock protein 70 expression. J Biol Chem. 1997;272(2):1402-11.

162. Arvans DL, Vavricka SR, Ren H, Musch MW, Kang L, Rocha FG, et al. Luminal bacterial flora determines physiological expression of intestinal epithelial cytoprotective heat shock proteins 25 and 72. Am J Physiol Gastrointest Liver Physiol. 2005;288(4):G696-704.

163. Petrof EO, Ciancio MJ, Chang EB. Role and regulation of intestinal epithelial heat shock proteins in health and disease. Chin J Dig Dis. 2004;5(2):45-50.

164. Kojima K, Musch MW, Ren H, Boone DL, Hendrickson BA, Ma A, et al. Enteric flora and lymphocyte-derived cytokines determine expression of heat shock proteins in mouse colonic epithelial cells. Gastroenterology. 2003;124(5):1395-407.

165. Schulz S, Wong RJ, Jang KY, Kalish F, Chisholm KM, Zhao H, et al. Heme oxygenase-1 deficiency promotes the development of necrotizing enterocolitis-like intestinal injury in a newborn mouse model. Am J Physiol Gastrointest Liver Physiol. 2013;304(11):G991-G1001.

166. Naidu S, Wijayanti N, Santoso S, Kietzmann T, Immenschuh S. An atypical NF-kappa B-regulated pathway mediates phorbol ester-dependent heme oxygenase-1 gene activation in monocytes. J Immunol. 2008;181(6):4113-23.

167. Halpern MD, Khailova L, Molla-Hosseini D, Arganbright K, Reynolds C, Yajima M, et al. Decreased development of necrotizing enterocolitis in IL-18-deficient mice. Am J Physiol Gastrointest Liver Physiol. 2008;294(1):G20-6.

168. Halpern MD, Weitkamp JH, Mount Patrick SK, Dobrenen HJ, Khailova L, Correa H, et al. Apical sodium-dependent bile acid transporter upregulation is associated with necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol. 2010;299(3):G623-31.

169. Hegazi RA, Rao KN, Mayle A, Sepulveda AR, Otterbein LE, Plevy SE. Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1-dependent pathway. J Exp Med. 2005;202(12):1703-13.

170. Chung SW, Liu X, Macias AA, Baron RM, Perrella MA. Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. J Clin Invest. 2008;118(1):239-47.

171. Giris M, Erbil Y, Oztezcan S, Olgac V, Barbaros U, Deveci U, et al. The effect of heme oxygenase-1 induction by glutamine on radiation-induced intestinal damage: the effect of heme oxygenase-1 on radiation enteritis. Am J Surg. 2006;191(4):503-9.

172. Naito Y, Takagi T, Yoshikawa T. Heme oxygenase-1: a new therapeutic target for inflammatory bowel disease. Aliment Pharmacol Ther. 2004;20 Suppl 1:177-84.

173. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res. 2003;92(8):827-39.

174. Gao Q, Meijer MJ, Kubben FJ, Sier CF, Kruidenier L, van Duijn W, et al. Expression of matrix metalloproteinases-2 and -9 in intestinal tissue of patients with inflammatory bowel diseases. Dig Liver Dis. 2005;37(8):584-92.

175. Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta. 2000;1477(1-2):267-83.

176. Al-Dasooqi N. Matrix metalloproteinases and gut toxicity following cytotoxic cancer therapy. Curr Opin Support Palliat Care. 2014;8(2):164-9.

177. Al-Dasooqi N, Gibson RJ, Bowen JM, Keefe DM. Matrix metalloproteinases: key regulators in the pathogenesis of chemotherapy-induced mucositis? Cancer Chemother Pharmacol. 2009;64(1):1-9.

178. Tobar N, Villar V, Santibanez JF. ROS-NFkappaB mediates TGF-beta1-induced expression of urokinase-type plasminogen activator, matrix metalloproteinase-9 and cell invasion. Mol Cell Biochem. 2010;340(1-2):195-202.

179. Garg P, Vijay-Kumar M, Wang L, Gewirtz AT, Merlin D, Sitaraman SV. Matrix metalloproteinase-9-mediated tissue injury overrides the protective effect of matrix metalloproteinase-2 during colitis. Am J Physiol Gastrointest Liver Physiol. 2009;296(2):G175-84.

180. Ganea E, Trifan M, Laslo AC, Putina G, Cristescu C. Matrix metalloproteinases: useful and deleterious. Biochem Soc Trans. 2007;35(Pt 4):689-91.

181. Milas L, Stephens LC, Meyn RE. Relation of apoptosis to cancer therapy. In Vivo. 1994;8(5):665-73.

182. Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med. 1997;185(8):1481-6.

183. Proskuryakov SY, Konoplyannikov AG, Gabai VL. Necrosis: a specific form of programmed cell death? Exp Cell Res. 2003;283(1):1-16.

184. Ravagnan L, Roumier T, Kroemer G. Mitochondria, the killer organelles and their weapons. J Cell Physiol. 2002;192(2):131-7.

185. Fehrenbach E, Northoff H. Free radicals, exercise, apoptosis, and heat shock proteins. Exerc Immunol Rev. 2001;7:66-89.

186. Zhang C, Liu J, Pan H, Yang X, Bian K. [Mitochondrial dysfunction induced by excessive ROS/RNS-metabolic cardiovascular disease and traditional Chinese medicines intervention]. Zhongguo Zhong Yao Za Zhi. 2011;36(17):2423-8.

187. Graf D, Bode JG, Haussinger D. Caspases and receptor cleavage. Arch Biochem Biophys. 2007;462(2):162-70.

188. Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Cytochrome c release from mitochondria proceeds by a two-step process. Proc Natl Acad Sci U S A. 2002;99(3):1259-63.

189. Michel H, Behr J, Harrenga A, Kannt A. Cytochrome c oxidase: structure and spectroscopy. Annu Rev Biophys Biomol Struct. 1998;27:329-56.

190. Jiang X, Wang X. Cytochrome C-mediated apoptosis. Annu Rev Biochem. 2004;73:87-106.

191. Adrain C, Martin SJ. Cell biology. Double knockout blow for caspases. Science. 2006;311(5762):785-6.

192. Wurstle ML, Laussmann MA, Rehm M. The central role of initiator caspase-9 in apoptosis signal transduction and the regulation of its activation and activity on the apoptosome. Exp Cell Res. 2012;318(11):1213-20.

193. Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem. 1999;68:383-424.

194. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. Mol Cell. 2010;37(3):299-310.

195. Morales J, Li L, Fattah FJ, Dong Y, Bey EA, Patel M, et al. Review of poly (ADP-ribose) polymerase (PARP) mechanisms of action and rationale for targeting in cancer and other diseases. Crit Rev Eukaryot Gene Expr. 2014;24(1):15-28.

196. D'Amours D, Sallmann FR, Dixit VM, Poirier GG. Gain-of-function of poly(ADP-ribose) polymerase-1 upon cleavage by apoptotic proteases: implications for apoptosis. J Cell Sci. 2001;114(Pt 20):3771-8.

197. Brune B, von Knethen A, Sandau KB. Nitric oxide (NO): an effector of apoptosis. Cell Death Differ. 1999;6(10):969-75.

198. Brown GC. Nitric oxide and mitochondrial respiration. Biochim Biophys Acta. 1999;1411(2-3):351-69.

199. Kolb JP. Mechanisms involved in the pro- and anti-apoptotic role of NO in human leukemia. Leukemia. 2000;14(9):1685-94.

200. Radi R, Rodriguez M, Castro L, Telleri R. Inhibition of mitochondrial electron transport by peroxynitrite. Arch Biochem Biophys. 1994;308(1):89-95.

201. Mannick JB, Hausladen A, Liu L, Hess DT, Zeng M, Miao QX, et al. Fas-induced caspase denitrosylation. Science. 1999;284(5414):651-4.

202. Brune B, von Knethen A, Sandau KB. Nitric oxide and its role in apoptosis. Eur J Pharmacol. 1998;351(3):261-72.

203. Choi BM, Pae HO, Jang SI, Kim YM, Chung HT. Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. J Biochem Mol Biol. 2002;35(1):116-26.

204. Taylor EL, Megson IL, Haslett C, Rossi AG. Nitric oxide: a key regulator of myeloid inflammatory cell apoptosis. Cell Death Differ. 2003;10(4):418-30.

205. Potten CS, Wilson JW, Booth C. Regulation and significance of apoptosis in the stem cells of the gastrointestinal epithelium. Stem Cells. 1997;15(2):82-93.

206. Zhang C, Sheng ZY, Hu S, Gao JC, Yu S, Liu Y. The influence of apoptosis of mucosal epithelial cells on intestinal barrier integrity after scald in rats. Burns. 2002;28(8):731-7.

207. Jin W, Wang H, Ji Y, Hu Q, Yan W, Chen G, et al. Increased intestinal inflammatory response and gut barrier dysfunction in Nrf2-deficient mice after traumatic brain injury. Cytokine. 2008;44(1):135-40.

208. Southan GJ, Szabo C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. Biochem Pharmacol. 1996;51(4):383-94.

209. Janakiram NB, Rao CV. iNOS-selective inhibitors for cancer prevention: promise and progress. Future Med Chem. 2012;4(17):2193-204.

210. Nilsson BO. Biological effects of aminoguanidine: an update. Inflamm Res. 1999;48(10):509-15.

211. Misko TP, Moore WM, Kasten TP, Nickols GA, Corbett JA, Tilton RG, et al. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. Eur J Pharmacol. 1993;233(1):119-25.

212. Corbett JA, McDaniel ML. The Use of Aminoguanidine, a Selective iNOS Inhibitor, to Evaluate the Role of Nitric Oxide in the Development of Autoimmune Diabetes. Methods. 1996;10(1):21-30.

213. Al-Majed AA, Khattab M, Raza M, Al-Shabanah OA, Mostafa AM. Potentiation of diclofenac-induced anti-inflammatory response by aminoguanidine in carrageenan-

induced acute inflammation in rats: the role of nitric oxide. Inflamm Res. 2003;52(9):378-82.

214. Courderot-Masuyer C, Dalloz F, Maupoil V, Rochette L. Antioxidant properties of aminoguanidine. Fundam Clin Pharmacol. 1999;13(5):535-40.

215. Burcham PC, Kaminskas LM, Fontaine FR, Petersen DR, Pyke SM. Aldehyde-sequestering drugs: tools for studying protein damage by lipid peroxidation products. Toxicology. 2002;181-182:229-36.

216. Brownlee M, Vlassara H, Kooney A, Ulrich P, Cerami A. Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. Science. 1986;232(4758):1629-32.

217. Kurose I, Higuchi H, Yonei Y, Ebinuma H, Watanabe N, Hokari R, et al. Rat Kupffer cell-derived nitric oxide suppresses proliferation and induces apoptosis of syngeneic hepatoma cells. Gastroenterology. 1996;111(4):1058-70.

218. Unno N, Wang H, Menconi MJ, Tytgat SH, Larkin V, Smith M, et al. Inhibition of inducible nitric oxide synthase ameliorates endotoxin-induced gut mucosal barrier dysfunction in rats. Gastroenterology. 1997;113(4):1246-57.

219. Bosca MM, Anon R, Mayordomo E, Villagrasa R, Balza N, Amoros C, et al. Methotrexate induced sprue-like syndrome. World J Gastroenterol. 2008;14(45):7009-11.

220. Mao Y, Kasravi B, Nobaek S, Wang LQ, Adawi D, Roos G, et al. Pectin-supplemented enteral diet reduces the severity of methotrexate induced enterocolitis in rats. Scand J Gastroenterol. 1996;31(6):558-67.

221. Miyazono Y, Gao F, Horie T. Oxidative stress contributes to methotrexate-induced small intestinal toxicity in rats. Scand J Gastroenterol. 2004;39(11):1119-27.

222. Chang CJ, Lin JF, Chang HH, Lee GA, Hung CF. Lutein protects against methotrexate-induced and reactive oxygen species-mediated apoptotic cell injury of IEC-6 cells. PLoS One. 2013;8(9):e72553.

223. Koppelmann T, Pollak Y, Mogilner J, Bejar J, Coran AG, Sukhotnik I. Reversal of severe methotrexate-induced intestinal damage using enteral n-3 fatty acids. Br J Nutr. 2013;109(1):89-98.

224. Kolli VK, Abraham P, Isaac B, Kasthuri N. Preclinical efficacy of melatonin to reduce methotrexate-induced oxidative stress and small intestinal damage in rats. Dig Dis Sci. 2013;58(4):959-69.

225. Leitao RF, Brito GA, Oria RB, Braga-Neto MB, Bellaguarda EA, Silva JV, et al. Role of inducible nitric oxide synthase pathway on methotrexate-induced intestinal mucositis in rodents. BMC Gastroenterol. 2011;11:90.

226. Abbasi M, Alizadeh R, Abolhassani F, Amidi F, Ragerdi KI, Fazelipour S, et al. Effect of aminoguanidine in sperm DNA fragmentation in varicocelized rats: role of nitric oxide. Reprod Sci. 2011;18(6):545-50.

227. Chiu CJ, McArdle AH, Brown R, Scott HJ, Gurd FN. Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. Arch Surg. 1970;101(4):478-83.

228. Young SL, Lessey BA, Fritz MA, Meyer WR, Murray MJ, Speckman PL, et al. In vivo and in vitro evidence suggest that HB-EGF regulates endometrial expression of human decay-accelerating factor. J Clin Endocrinol Metab. 2002;87(3):1368-75.

229. Noursadeghi M, Tsang J, Haustein T, Miller RF, Chain BM, Katz DR. Quantitative imaging assay for NF-kappaB nuclear translocation in primary human macrophages. J Immunol Methods. 2008;329(1-2):194-200.

230. Wong RG, Myrtle JF, Tsai HC, Norman AW. Studies on calciferol metabolism. V. The occurrence and biological activity of 1,25-dihydroxy-vitamin D 3 in bone. J Biol Chem. 1972;247(18):5728-35.

231. Morton RL, Ikle D, White CW. Loss of lung mitochondrial aconitase activity due to hyperoxia in bronchopulmonary dysplasia in primates. Am J Physiol. 1998;274(1 Pt 1):L127-33.

232. Jacobus WE, Lehninger AL. Creatine kinase of rat heart mitochondria. Coupling of creatine phosphorylation to electron transport. J Biol Chem. 1973;248(13):4803-10.

233. Hamelin M, Mary J, Vostry M, Friguet B, Bakala H. Glycation damage targets glutamate dehydrogenase in the rat liver mitochondrial matrix during aging. FEBS J. 2007;274(22):5949-61.

234. Shapira E, Ben-Yoseph Y, Eyal FG, Russell A. Enzymatically inactive red cell carbonic anhydrase B in a family with renal tubular acidosis. J Clin Invest. 1974;53(1):59-63.

235. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265-75.

236. Pignatelli B, Li CQ, Boffetta P, Chen Q, Ahrens W, Nyberg F, et al. Nitrated and oxidized plasma proteins in smokers and lung cancer patients. Cancer Res. 2001;61(2):778-84.

237. Matassov D, Kagan T, Leblanc J, Sikorska M, Zakeri Z. Measurement of apoptosis by DNA fragmentation. Methods Mol Biol. 2004;282:1-17.

238. Duncan M, Grant G. Oral and intestinal mucositis - causes and possible treatments. Aliment Pharmacol Ther. 2003;18(9):853-74.

239. Leitao RF, Ribeiro RA, Bellaguarda EA, Macedo FD, Silva LR, Oria RB, et al. Role of nitric oxide on pathogenesis of 5-fluorouracil induced experimental oral mucositis in hamster. Cancer Chemother Pharmacol. 2007;59(5):603-12.

240. Kamat JP. Peroxynitrite: a potent oxidizing and nitrating agent. Indian J Exp Biol. 2006;44(6):436-47.

241. Liu S, Adcock IM, Old RW, Barnes PJ, Evans TW. Lipopolysaccharide treatment in vivo induces widespread tissue expression of inducible nitric oxide synthase mRNA. Biochem Biophys Res Commun. 1993;196(3):1208-13.

242. Ohmori H, Kanayama N. Immunogenicity of an inflammation-associated product, tyrosine nitrated self-proteins. Autoimmun Rev. 2005;4(4):224-9.

243. Greenacre SA, Ischiropoulos H. Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. Free Radic Res. 2001;34(6):541-81.

244. de Koning BA, van Dieren JM, Lindenbergh-Kortleve DJ, van der Sluis M, Matsumoto T, Yamaguchi K, et al. Contributions of mucosal immune cells to methotrexate-induced mucositis. Int Immunol. 2006;18(6):941-9.

245. Guo W, Adachi T, Matsui R, Xu S, Jiang B, Zou MH, et al. Quantitative assessment of tyrosine nitration of manganese superoxide dismutase in angiotensin II-infused rat kidney. Am J Physiol Heart Circ Physiol. 2003;285(4):H1396-403.

246. Wallace JL. Cooperative modulation of gastrointestinal mucosal defence by prostaglandins and nitric oxide. Clin Invest Med. 1996;19(5):346-51.

247. Kubes P, McCafferty DM. Nitric oxide and intestinal inflammation. Am J Med. 2000;109(2):150-8.

248. Lobo SM, Soriano FG, Barbeiro DF, De Backer D, Sun Q, Tu Z, et al. Effects of dobutamine on gut mucosal nitric oxide production during endotoxic shock in rabbits. Med Sci Monit. 2009;15(2):BR37-42.

249. Nathan C. Inducible nitric oxide synthase: what difference does it make? J Clin Invest. 1997;100(10):2417-23.

250. McCafferty DM, Mudgett JS, Swain MG, Kubes P. Inducible nitric oxide synthase plays a critical role in resolving intestinal inflammation. Gastroenterology. 1997;112(3):1022-7.

251. Millar TM, Stevens CR, Benjamin N, Eisenthal R, Harrison R, Blake DR. Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. FEBS Lett. 1998;427(2):225-8.

252. Sessa WC. The nitric oxide synthase family of proteins. J Vasc Res. 1994;31(3):131-43.

253. Lima-Junior RC, Figueiredo AA, Freitas HC, Melo ML, Wong DV, Leite CA, et al. Involvement of nitric oxide on the pathogenesis of irinotecan-induced intestinal mucositis: role of cytokines on inducible nitric oxide synthase activation. Cancer Chemother Pharmacol. 2012;69(4):931-42.

254. Feng A, Zhou G, Yuan X, Huang X, Zhang Z, Zhang T. Inhibitory effect of baicalin on iNOS and NO expression in intestinal mucosa of rats with acute endotoxemia. PLoS One. 2013;8(12):e80997.

255. Talukder MJ, Harada E. Bovine lactoferrin protects lipopolysaccharide-induced diarrhea modulating nitric oxide and prostaglandin E2 in mice. Can J Physiol Pharmacol. 2007;85(2):200-8.

256. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med. 1993;329(27):2002-12.

257. Farrell AJ, Blake DR. Nitric oxide. Ann Rheum Dis. 1996;55(1):7-20.

258. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. 1996;271(5 Pt 1):C1424-37.

259. Kanwar JR, Kanwar RK, Burrow H, Baratchi S. Recent advances on the roles of NO in cancer and chronic inflammatory disorders. Curr Med Chem. 2009;16(19):2373-94.

260. Han KK, Martinage A. Post-translational chemical modification(s) of proteins. Int J Biochem. 1992;24(1):19-28.

261. Sokolovsky M, Riordan JF, Vallee BL. Tetranitromethane. A reagent for the nitration of tyrosyl residues in proteins. Biochemistry. 1966;5(11):3582-9.

262. Gaut JP, Byun J, Tran HD, Heinecke JW. Artifact-free quantification of free 3-chlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionization gas chromatography mass spectrometry and liquid chromatography-electrospray ionization tandem mass spectrometry. Anal Biochem. 2002;300(2):252-9.

263. Tohgi H, Abe T, Yamazaki K, Murata T, Ishizaki E, Isobe C. Alterations of 3-nitrotyrosine concentration in the cerebrospinal fluid during aging and in patients with Alzheimer's disease. Neurosci Lett. 1999;269(1):52-4.

264. Palazzolo-Ballance AM, Suquet C, Hurst JK. Pathways for intracellular generation of oxidants and tyrosine nitration by a macrophage cell line. Biochemistry. 2007;46(25):7536-48.

265. Lohinai Z, Stachlewitz R, Virag L, Szekely AD, Hasko G, Szabo C. Evidence for reactive nitrogen species formation in the gingivomucosal tissue. J Dent Res. 2001;80(2):470-5.

266. Mohiuddin I, Chai H, Lin PH, Lumsden AB, Yao Q, Chen C. Nitrotyrosine and chlorotyrosine: clinical significance and biological functions in the vascular system. J Surg Res. 2006;133(2):143-9.

267. Oury TD, Tatro L, Ghio AJ, Piantadosi CA. Nitration of tyrosine by hydrogen peroxide and nitrite. Free Radic Res. 1995;23(6):537-47.

268. Heijnen HF, van Donselaar E, Slot JW, Fries DM, Blachard-Fillion B, Hodara R, et al. Subcellular localization of tyrosine-nitrated proteins is dictated by reactive oxygen species generating enzymes and by proximity to nitric oxide synthase. Free Radic Biol Med. 2006;40(11):1903-13.

269. Denicola A, Souza JM, Radi R. Diffusion of peroxynitrite across erythrocyte membranes. Proc Natl Acad Sci U S A. 1998;95(7):3566-71.

270. Ischiropoulos H. Biological selectivity and functional aspects of protein tyrosine nitration. Biochem Biophys Res Commun. 2003;305(3):776-83.

271. Cover C, Mansouri A, Knight TR, Bajt ML, Lemasters JJ, Pessayre D, et al. Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. J Pharmacol Exp Ther. 2005;315(2):879-87.

272. Liu B, Tewari AK, Zhang L, Green-Church KB, Zweier JL, Chen YR, et al. Proteomic analysis of protein tyrosine nitration after ischemia reperfusion injury: mitochondria as the major target. Biochim Biophys Acta. 2009;1794(3):476-85.

273. Hinson JA, Michael SL, Ault SG, Pumford NR. Western blot analysis for nitrotyrosine protein adducts in livers of saline-treated and acetaminophen-treated mice. Toxicol Sci. 2000;53(2):467-73.

274. Levrand S, Vannay-Bouchiche C, Pesse B, Pacher P, Feihl F, Waeber B, et al. Peroxynitrite is a major trigger of cardiomyocyte apoptosis in vitro and in vivo. Free Radic Biol Med. 2006;41(6):886-95.

275. Wang G, Wang J, Luo X, Ansari GA, Khan MF. Nitrosative stress and nitrated proteins in trichloroethene-mediated autoimmunity. PLoS One. 2014;9(6):e98660.

276. Fan X, Wang J, Soman KV, Ansari GA, Khan MF. Aniline-induced nitrosative stress in rat spleen: proteomic identification of nitrated proteins. Toxicol Appl Pharmacol. 2011;255(1):103-12.

277. Vanderzant CW, Beydoun AA, Domer PA, Hood TW, Abou-Khalil BW. Polarity reversal of N20 and P23 somatosensory evoked potentials between scalp and depth recordings. Electroencephalogr Clin Neurophysiol. 1991;78(3):234-9.

278. Zou MH. Peroxynitrite and protein tyrosine nitration of prostacyclin synthase. Prostaglandins Other Lipid Mediat. 2007;82(1-4):119-27.

279. Mehl M, Daiber A, Herold S, Shoun H, Ullrich V. Peroxynitrite reaction with heme proteins. Nitric Oxide. 1999;3(2):142-52.

280. Bartesaghi S, Valez V, Trujillo M, Peluffo G, Romero N, Zhang H, et al. Mechanistic studies of peroxynitrite-mediated tyrosine nitration in membranes using the hydrophobic probe N-t-BOC-L-tyrosine tert-butyl ester. Biochemistry. 2006;45(22):6813-25.

281. Aulak KS, Koeck T, Crabb JW, Stuehr DJ. Proteomic method for identification of tyrosine-nitrated proteins. Methods Mol Biol. 2004;279:151-65.

282. MacMillan-Crow LA, Thompson JA. Immunoprecipitation of nitrotyrosine-containing proteins. Methods Enzymol. 1999;301:135-45.

283. Helman M, Givol D. Isolation of nitrotyrosine-containing peptides by using an insoluble-antibody column. Biochem J. 1971;125(4):971-4.

284. Zhang Q, Qian WJ, Knyushko TV, Clauss TR, Purvine SO, Moore RJ, et al. A method for selective enrichment and analysis of nitrotyrosine-containing peptides in complex proteome samples. J Proteome Res. 2007;6(6):2257-68.

285. Aulak KS, Miyagi M, Yan L, West KA, Massillon D, Crabb JW, et al. Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. Proc Natl Acad Sci U S A. 2001;98(21):12056-61.

286. Raisanen SR, Lehenkari P, Tasanen M, Rahkila P, Harkonen PL, Vaananen HK. Carbonic anhydrase III protects cells from hydrogen peroxide-induced apoptosis. FASEB J. 1999;13(3):513-22.

287. Gow AJ, Duran D, Malcolm S, Ischiropoulos H. Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. FEBS Lett. 1996;385(1-2):63-6.

288. Gole MD, Souza JM, Choi I, Hertkorn C, Malcolm S, Foust RF, 3rd, et al. Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol. 2000;278(5):L961-7.

289. Monteiro HP, Arai RJ, Travassos LR. Protein tyrosine phosphorylation and protein tyrosine nitration in redox signaling. Antioxid Redox Signal. 2008;10(5):843-89.

290. Murray J, Taylor SW, Zhang B, Ghosh SS, Capaldi RA. Oxidative damage to mitochondrial complex I due to peroxynitrite: identification of reactive tyrosines by mass spectrometry. J Biol Chem. 2003;278(39):37223-30.

291. Radi R, Cassina A, Hodara R. Nitric oxide and peroxynitrite interactions with mitochondria. Biol Chem. 2002;383(3-4):401-9.

292. Shiva S, Brookes PS, Patel RP, Anderson PG, Darley-Usmar VM. Nitric oxide partitioning into mitochondrial membranes and the control of respiration at cytochrome c oxidase. Proc Natl Acad Sci U S A. 2001;98(13):7212-7.

293. Haynes V, Traaseth NJ, Elfering S, Fujisawa Y, Giulivi C. Nitration of specific tyrosines in FoF1 ATP synthase and activity loss in aging. Am J Physiol Endocrinol Metab. 2010;298(5):E978-87.

294. Reed TT, Pierce WM, Jr., Turner DM, Markesbery WR, Butterfield DA. Proteomic identification of nitrated brain proteins in early Alzheimer's disease inferior parietal lobule. J Cell Mol Med. 2009;13(8B):2019-29.

295. Abdelmegeed MA, Song BJ. Functional roles of protein nitration in acute and chronic liver diseases. Oxid Med Cell Longev. 2014;2014:149627.

296. Shenouda SK, Lord KC, McIlwain E, Lucchesi PA, Varner KJ. Ecstasy produces left ventricular dysfunction and oxidative stress in rats. Cardiovasc Res. 2008;79(4):662-70.

297. Zhan X, Du Y, Crabb JS, Gu X, Kern TS, Crabb JW. Targets of tyrosine nitration in diabetic rat retina. Mol Cell Proteomics. 2008;7(5):864-74.

298. Lam PY, Yin F, Hamilton RT, Boveris A, Cadenas E. Elevated neuronal nitric oxide synthase expression during ageing and mitochondrial energy production. Free Radic Res. 2009;43(5):431-9.

299. Tortora V, Quijano C, Freeman B, Radi R, Castro L. Mitochondrial aconitase reaction with nitric oxide, S-nitrosoglutathione, and peroxynitrite: mechanisms and relative contributions to aconitase inactivation. Free Radic Biol Med. 2007;42(7):1075-88.

300. Alen C, Sonenshein AL. Bacillus subtilis aconitase is an RNA-binding protein. Proc Natl Acad Sci U S A. 1999;96(18):10412-7.

301. Han D, Canali R, Garcia J, Aguilera R, Gallaher TK, Cadenas E. Sites and mechanisms of aconitase inactivation by peroxynitrite: modulation by citrate and glutathione. Biochemistry. 2005;44(36):11986-96.

302. Boquist L, Ericsson I, Lorentzon R, Nelson L. Alterations in mitochondrial aconitase activity and respiration, and in concentration of citrate in some organs of mice with experimental or genetic diabetes. FEBS Lett. 1985;183(1):173-6.

303. Raza H, Prabu SK, John A, Avadhani NG. Impaired mitochondrial respiratory functions and oxidative stress in streptozotocin-induced diabetic rats. Int J Mol Sci. 2011;12(5):3133-47.

304. Lin G, Brownsey RW, MacLeod KM. Regulation of mitochondrial aconitase by phosphorylation in diabetic rat heart. Cell Mol Life Sci. 2009;66(5):919-32.

305. Satav JG, Katyare SS. Effect of streptozotocin-induced diabetes on oxidative energy metabolism in rat liver mitochondria-A comparative study of early and late effects. Indian J Clin Biochem. 2004;19(2):23-31.

306. Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell. 2008;13(6):472-82.

307. Zhang Y, Lu N, Gao Z. Hemin-H2O2-NO2(-) induced protein oxidation and tyrosine nitration are different from those of SIN-1: a study on glutamate dehydrogenase nitrative/oxidative modification. Int J Biochem Cell Biol. 2009;41(4):907-15.

308. Elfering SL, Haynes VL, Traaseth NJ, Ettl A, Giulivi C. Aspects, mechanism, and biological relevance of mitochondrial protein nitration sustained by mitochondrial nitric oxide synthase. Am J Physiol Heart Circ Physiol. 2004;286(1):H22-9.

309. Wyss M, Smeitink J, Wevers RA, Wallimann T. Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. Biochim Biophys Acta. 1992;1102(2):119-66.

310. Kanski J, Schoneich C. Protein nitration in biological aging: proteomic and tandem mass spectrometric characterization of nitrated sites. Methods Enzymol. 2005;396:160-71.

311. Mihm MJ, Bauer JA. Peroxynitrite-induced inhibition and nitration of cardiac myofibrillar creatine kinase. Biochimie. 2002;84(10):1013-9.

312. Kanski J, Alterman MA, Schoneich C. Proteomic identification of age-dependent protein nitration in rat skeletal muscle. Free Radic Biol Med. 2003;35(10):1229-39.

313. Seija M, Baccino C, Nin N, Sanchez-Rodriguez C, Granados R, Ferruelo A, et al. Role of peroxynitrite in sepsis-induced acute kidney injury in an experimental model of sepsis in rats. Shock. 2012;38(4):403-10.

314. Kanski J, Behring A, Pelling J, Schoneich C. Proteomic identification of 3-nitrotyrosine-containing rat cardiac proteins: effects of biological aging. Am J Physiol Heart Circ Physiol. 2005;288(1):H371-81.

315. Jenny NS. Inflammation in aging: cause, effect, or both? Discov Med. 2012;13(73):451-60.

316. Oberley LW, Buettner GR. Role of superoxide dismutase in cancer: a review. Cancer Res. 1979;39(4):1141-9.

317. Marklund SL. Human copper-containing superoxide dismutase of high molecular weight. Proc Natl Acad Sci U S A. 1982;79(24):7634-8.

318. Quijano C, Hernandez-Saavedra D, Castro L, McCord JM, Freeman BA, Radi R. Reaction of peroxynitrite with Mn-superoxide dismutase. Role of the metal center in decomposition kinetics and nitration. J Biol Chem. 2001;276(15):11631-8.

319. Yamakura F, Kawasaki H. Post-translational modifications of superoxide dismutase. Biochim Biophys Acta. 2010;1804(2):318-25.

320. Dowling EJ, Chander CL, Claxson AW, Lillie C, Blake DR. Assessment of a human recombinant manganese superoxide dismutase in models of inflammation. Free Radic Res Commun. 1993;18(5):291-8.

321. Redondo-Horcajo M, Romero N, Martinez-Acedo P, Martinez-Ruiz A, Quijano C, Lourenco CF, et al. Cyclosporine A-induced nitration of tyrosine 34 MnSOD in endothelial cells: role of mitochondrial superoxide. Cardiovasc Res. 2010;87(2):356-65.

322. Ueda J, Starr ME, Takahashi H, Du J, Chang LY, Crapo JD, et al. Decreased pulmonary extracellular superoxide dismutase during systemic inflammation. Free Radic Biol Med. 2008;45(6):897-904.

323. Bayir H, Kagan VE, Clark RS, Janesko-Feldman K, Rafikov R, Huang Z, et al. Neuronal NOS-mediated nitration and inactivation of manganese superoxide dismutase in brain after experimental and human brain injury. J Neurochem. 2007;101(1):168-81.

324. Comhair SA, Xu W, Ghosh S, Thunnissen FB, Almasan A, Calhoun WJ, et al. Superoxide dismutase inactivation in pathophysiology of asthmatic airway remodeling and reactivity. Am J Pathol. 2005;166(3):663-74.

325. Fridovich I. Superoxide anion radical (O2-.), superoxide dismutases, and related matters. J Biol Chem. 1997;272(30):18515-7.

326. Mao GD, Thomas PD, Lopaschuk GD, Poznansky MJ. Superoxide dismutase (SOD)-catalase conjugates. Role of hydrogen peroxide and the Fenton reaction in SOD toxicity. J Biol Chem. 1993;268(1):416-20.

327. Tanaka M, Bateman R, Rauh D, Vaisberg E, Ramachandani S, Zhang C, et al. An unbiased cell morphology-based screen for new, biologically active small molecules.

PLoS Biol. 2005;3(5):e128. 328. Liska DJ. The detoxification enzyme systems. Altern Med Rev. 1998;3(3):187-98.

329. Baek IJ, Seo DS, Yon JM, Lee SR, Jin Y, Nahm SS, et al. Tissue expression and cellular localization of phospholipid hydroperoxide glutathione peroxidase (PHGPx) mRNA in male mice. J Mol Histol. 2007;38(3):237-44.

330. Vaisberg CN, Jelezarsky LV, Dishlianova B, Chaushev TA. Activity, substrate detection and immunolocalization of glutathione peroxidase (GPx) in bovine reproductive organs and semen. Theriogenology. 2005;64(2):416-28.

331. Sies H, Sharov VS, Klotz LO, Briviba K. Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase. J Biol Chem. 1997;272(44):27812-7.

332. Tham DM, Whitin JC, Kim KK, Zhu SX, Cohen HJ. Expression of extracellular glutathione peroxidase in human and mouse gastrointestinal tract. Am J Physiol. 1998;275(6 Pt 1):G1463-71.

333. Florian S, Krehl S, Loewinger M, Kipp A, Banning A, Esworthy S, et al. Loss of GPx2 increases apoptosis, mitosis, and GPx1 expression in the intestine of mice. Free Radic Biol Med. 2010;49(11):1694-702.

334. Grzelak A, Soszynski M, Bartosz G. Inactivation of antioxidant enzymes by peroxynitrite. Scand J Clin Lab Invest. 2000;60(4):253-8.

335. Hoensch H, Peters WH, Roelofs HM, Kirch W. Expression of the glutathione enzyme system of human colon mucosa by localisation, gender and age. Curr Med Res Opin. 2006;22(6):1075-83.

336. Ji Y, Neverova I, Van Eyk JE, Bennett BM. Nitration of tyrosine 92 mediates the activation of rat microsomal glutathione s-transferase by peroxynitrite. J Biol Chem. 2006;281(4):1986-91.

337. Wong PS, Eiserich JP, Reddy S, Lopez CL, Cross CE, van der Vliet A. Inactivation of glutathione S-transferases by nitric oxide-derived oxidants: exploring a role for tyrosine nitration. Arch Biochem Biophys. 2001;394(2):216-28.

338. Chatterjee B, Nath TS, Roy AK. Differential regulation of the messenger RNA for three major senescence marker proteins in male rat liver. J Biol Chem. 1981;256(12):5939-41.

339. Zimmerman UJ, Wang P, Zhang X, Bogdanovich S, Forster R. Anti-oxidative response of carbonic anhydrase III in skeletal muscle. IUBMB Life. 2004;56(6):343-7.

340. Lii CK, Chai YC, Zhao W, Thomas JA, Hendrich S. S-thiolation and irreversible oxidation of sulfhydryls on carbonic anhydrase III during oxidative stress: a method for

studying protein modification in intact cells and tissues. Arch Biochem Biophys. 1994;308(1):231-9.

341. Laroux FS, Pavlick KP, Hines IN, Kawachi S, Harada H, Bharwani S, et al. Role of nitric oxide in inflammation. Acta Physiol Scand. 2001;173(1):113-8.

342. Li Y, Liu D, Liu Y, Li E, Wang H, Liu K, et al. Protein nitration promotes inducible nitric oxide synthase transcription mediated by NF-kappaB in high glucose-stimulated human lens epithelial cells. Mol Cell Endocrinol. 2013;370(1-2):78-86.

343. Park SW, Huq MD, Hu X, Wei LN. Tyrosine nitration on p65: a novel mechanism to rapidly inactivate nuclear factor-kappaB. Mol Cell Proteomics. 2005;4(3):300-9.

344. Logan RM, Gibson RJ, Bowen JM, Stringer AM, Sonis ST, Keefe DM. Characterisation of mucosal changes in the alimentary tract following administration of irinotecan: implications for the pathobiology of mucositis. Cancer Chemother Pharmacol. 2008;62(1):33-41.

345. Yamamoto Y, Gaynor RB. IkappaB kinases: key regulators of the NF-kappaB pathway. Trends Biochem Sci. 2004;29(2):72-9.

346. Pasparakis M. Regulation of tissue homeostasis by NF-kappaB signalling: implications for inflammatory diseases. Nat Rev Immunol. 2009;9(11):778-88.

347. Beg AA, Sha WC, Bronson RT, Baltimore D. Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. Genes Dev. 1995;9(22):2736-46.

348. Klement JF, Rice NR, Car BD, Abbondanzo SJ, Powers GD, Bhatt PH, et al. IkappaBalpha deficiency results in a sustained NF-kappaB response and severe widespread dermatitis in mice. Mol Cell Biol. 1996;16(5):2341-9.

349. Greten FR, Karin M. The IKK/NF-kappaB activation pathway-a target for prevention and treatment of cancer. Cancer Lett. 2004;206(2):193-9.

350. Chen C, Edelstein LC, Gelinas C. The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol Cell Biol. 2000;20(8):2687-95.

351. Catz SD, Johnson JL. Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. Oncogene. 2001;20(50):7342-51.

352. De Plaen IG, Tan XD, Chang H, Qu XW, Liu QP, Hsueh W. Intestinal NF-kappaB is activated, mainly as p50 homodimers, by platelet-activating factor. Biochim Biophys Acta. 1998;1392(2-3):185-92.

353. delaTorre A, Schroeder RA, Punzalan C, Kuo PC. Endotoxin-mediated S-nitrosylation of p50 alters NF-kappa B-dependent gene transcription in ANA-1 murine macrophages. J Immunol. 1999;162(7):4101-8.

354. Xie W, Tian Y. Xenobiotic receptor meets NF-kappaB, a collision in the small bowel. Cell Metab. 2006;4(3):177-8.

355. Chen F, Castranova V, Shi X, Demers LM. New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. Clin Chem. 1999;45(1):7-17.

356. Neurath MF, Becker C, Barbulescu K. Role of NF-kappaB in immune and inflammatory responses in the gut. Gut. 1998;43(6):856-60.

357. Mollace V, Muscoli C, Masini E, Cuzzocrea S, Salvemini D. Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors. Pharmacol Rev. 2005;57(2):217-52.

358. Hierholzer C, Harbrecht B, Menezes JM, Kane J, MacMicking J, Nathan CF, et al. Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. J Exp Med. 1998;187(6):917-28.

359. Umansky V, Hehner SP, Dumont A, Hofmann TG, Schirrmacher V, Droge W, et al. Co-stimulatory effect of nitric oxide on endothelial NF-kappaB implies a physiological self-amplifying mechanism. Eur J Immunol. 1998;28(8):2276-82.

360. Hotz-Behofsits C, Simpson RJ, Walley M, Bjarnason IT. Role of COX-2 in nonsteroidal anti-inflammatory drug enteropathy in rodents. Scand J Gastroenterol. 2010;45(7-8):822-7.

361. Loetscher H, Schlaeger EJ, Lahm HW, Pan YC, Lesslauer W, Brockhaus M. Purification and partial amino acid sequence analysis of two distinct tumor necrosis factor receptors from HL60 cells. J Biol Chem. 1990;265(33):20131-8.

362. Elewaut D, DiDonato JA, Kim JM, Truong F, Eckmann L, Kagnoff MF. NF-kappa B is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria. J Immunol. 1999;163(3):1457-66.

363. Jung HC, Eckmann L, Yang SK, Panja A, Fierer J, Morzycka-Wroblewska E, et al. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J Clin Invest. 1995;95(1):55-65.

364. Hamada K, Kakigawa N, Sekine S, Shitara Y, Horie T. Disruption of ZO-1/claudin-4 interaction in relation to inflammatory responses in methotrexate-induced intestinal mucositis. Cancer Chemother Pharmacol. 2013;72(4):757-65.

365. Soares PM, Mota JM, Souza EP, Justino PF, Franco AX, Cunha FQ, et al. Inflammatory intestinal damage induced by 5-fluorouracil requires IL-4. Cytokine. 2013;61(1):46-9.

366. Yeoh A, Gibson R, Yeoh E, Bowen J, Stringer A, Giam K, et al. Radiation therapy-induced mucositis: relationships between fractionated radiation, NF-kappaB, COX-1, and COX-2. Cancer Treat Rev. 2006;32(8):645-51.

367. Salvemini D, Settle SL, Masferrer JL, Seibert K, Currie MG, Needleman P. Regulation of prostaglandin production by nitric oxide; an in vivo analysis. Br J Pharmacol. 1995;114(6):1171-8.

368. Hotz-Behofsits CM, Walley MJ, Simpson R, Bjarnason IT. COX-1, COX-2 and the topical effect in NSAID-induced enteropathy. Inflammopharmacology. 2003;11(4):363-70.

369. Rocca B, FitzGerald GA. Cyclooxygenases and prostaglandins: shaping up the immune response. Int Immunopharmacol. 2002;2(5):603-30.

370. Kiyohara H, Egami H, Shibata Y, Murata K, Ohshima S, Ogawa M. Light microscopic immunohistochemical analysis of the distribution of group II phospholipase A2 in human digestive organs. J Histochem Cytochem. 1992;40(11):1659-64.

371. Olaison G, Sjodahl R, Tagesson C. Increased phospholipase A2 activity of Ileal mucosa in Crohn's disease. Digestion. 1988;41(3):136-41.

372. Bomalaski JS, Baker DG, Brophy L, Resurreccion NV, Spilberg I, Muniain M, et al. A phospholipase A2-activating protein (PLAP) stimulates human neutrophil aggregation and release of lysosomal enzymes, superoxide, and eicosanoids. J Immunol. 1989;142(11):3957-62.

373. Al-Dasooqi N, Wardill HR, Gibson RJ. Gastrointestinal mucositis: the role of MMP-tight junction interactions in tissue injury. Pathol Oncol Res. 2014;20(3):485-91.

374. Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol. 2004;4(8):617-29.

375. Kerkela E, Saarialho-Kere U. Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer. Exp Dermatol. 2003;12(2):109-25.

376. Van den Steen PE, Opdenakker G, Wormald MR, Dwek RA, Rudd PM. Matrix remodelling enzymes, the protease cascade and glycosylation. Biochim Biophys Acta. 2001;1528(2-3):61-73.

377. Castaneda FE, Walia B, Vijay-Kumar M, Patel NR, Roser S, Kolachala VL, et al. Targeted deletion of metalloproteinase 9 attenuates experimental colitis in mice: central role of epithelial-derived MMP. Gastroenterology. 2005;129(6):1991-2008.

378. Mikami Y, Dobschutz EV, Sommer O, Wellner U, Unno M, Hopt U, et al. Matrix metalloproteinase-9 derived from polymorphonuclear neutrophils increases gut barrier dysfunction and bacterial translocation in rat severe acute pancreatitis. Surgery. 2009;145(2):147-56.

379. Medina C, Santana A, Paz MC, Diaz-Gonzalez F, Farre E, Salas A, et al. Matrix metalloproteinase-9 modulates intestinal injury in rats with transmural colitis. J Leukoc Biol. 2006;79(5):954-62.

380. Santana A, Medina C, Paz-Cabrera MC, Diaz-Gonzalez F, Farre E, Salas A, et al. Attenuation of dextran sodium sulphate induced colitis in matrix metalloproteinase-9 deficient mice. World J Gastroenterol. 2006;12(40):6464-72.

381. Pedersen G, Saermark T, Kirkegaard T, Brynskov J. Spontaneous and cytokine induced expression and activity of matrix metalloproteinases in human colonic epithelium. Clin Exp Immunol. 2009;155(2):257-65.

382. Yamada S, Naito Y, Takagi T, Mizushima K, Horie R, Fukumoto K, et al. Rebamipide ameliorates indomethacin-induced small intestinal injury in rats via the inhibition of matrix metalloproteinases activity. J Gastroenterol Hepatol. 2012;27(12):1816-24.

383. Papp E, Nardai G, Soti C, Csermely P. Molecular chaperones, stress proteins and redox homeostasis. Biofactors. 2003;17(1-4):249-57.

384. Shang F, Taylor A. Ubiquitin-proteasome pathway and cellular responses to oxidative stress. Free Radic Biol Med. 2011;51(1):5-16.

385. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 1997;91(4):479-89.

386. Menoret A, Chaillot D, Callahan M, Jacquin C. Hsp70, an immunological actor playing with the intracellular self under oxidative stress. Int J Hyperthermia. 2002;18(6):490-505.

387. Zlatkovic J, Bernardi RE, Filipovic D. Protective effect of Hsp70i against chronic social isolation stress in the rat hippocampus. J Neural Transm. 2014;121(1):3-14.

388. Dokladny K, Moseley PL, Ma TY. Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. Am J Physiol Gastrointest Liver Physiol. 2006;290(2):G204-12.

389. Liao YF, Zhu W, Li DP, Zhu X. Heme oxygenase-1 and gut ischemia/reperfusion injury: A short review. World J Gastroenterol. 2013;19(23):3555-61.

390. Coeffier M, Le Pessot F, Leplingard A, Marion R, Lerebours E, Ducrotte P, et al. Acute enteral glutamine infusion enhances heme oxygenase-1 expression in human duodenal mucosa. J Nutr. 2002;132(9):2570-3.

391. Barton SG, Rampton DS, Winrow VR, Domizio P, Feakins RM. Expression of heat shock protein 32 (hemoxygenase-1) in the normal and inflamed human stomach and colon: an immunohistochemical study. Cell Stress Chaperones. 2003;8(4):329-34.

392. Guo JS, Cho CH, Wang JY, Koo MW. Expression and immunolocalization of heat shock proteins in the healing of gastric ulcers in rats. Scand J Gastroenterol. 2002;37(1):17-22.

393. Wang WP, Guo X, Koo MW, Wong BC, Lam SK, Ye YN, et al. Protective role of heme oxygenase-1 on trinitrobenzene sulfonic acid-induced colitis in rats. Am J Physiol Gastrointest Liver Physiol. 2001;281(2):G586-94.

394. Guo X, Shin VY, Cho CH. Modulation of heme oxygenase in tissue injury and its implication in protection against gastrointestinal diseases. Life Sci. 2001;69(25-26):3113-9.

395. Sheikh SZ, Hegazi RA, Kobayashi T, Onyiah JC, Russo SM, Matsuoka K, et al. An anti-inflammatory role for carbon monoxide and heme oxygenase-1 in chronic Th2-mediated murine colitis. J Immunol. 2011;186(9):5506-13.

396. Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol. 2003;24(8):449-55.

397. Chang CT, Ho TY, Lin H, Liang JA, Huang HC, Li CC, et al. 5-Fluorouracil induced intestinal mucositis via nuclear factor-kappaB activation by transcriptomic analysis and in vivo bioluminescence imaging. PLoS One. 2012;7(3):e31808.

398. Yasuda M, Kato S, Yamanaka N, Iimori M, Utsumi D, Kitahara Y, et al. Potential role of the NADPH oxidase NOX1 in the pathogenesis of 5-fluorouracil-induced intestinal mucositis in mice. Am J Physiol Gastrointest Liver Physiol. 2012;302(10):G1133-42.

399. Sacksteder CA, Qian WJ, Knyushko TV, Wang H, Chin MH, Lacan G, et al. Endogenously nitrated proteins in mouse brain: links to neurodegenerative disease. Biochemistry. 2006;45(26):8009-22.

400. Chaitanya GV, Steven AJ, Babu PP. PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. Cell Commun Signal. 2010;8:31.

401. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res. 1993;53(17):3976-85.

402. Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, et al. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell. 1995;81(5):801-9.

403. Lemaire C, Andreau K, Souvannavong V, Adam A. Inhibition of caspase activity induces a switch from apoptosis to necrosis. FEBS Lett. 1998;425(2):266-70.

404. Herceg Z, Wang ZQ. Failure of poly(ADP-ribose) polymerase cleavage by caspases leads to induction of necrosis and enhanced apoptosis. Mol Cell Biol. 1999;19(7):5124-33.

405. Gibson RJ, Bowen JM, Inglis MR, Cummins AG, Keefe DM. Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer. J Gastroenterol Hepatol. 2003;18(9):1095-100.

406. Yasuda M, Kato S, Yamanaka N, Iimori M, Matsumoto K, Utsumi D, et al. 5-HT(3) receptor antagonists ameliorate 5-fluorouracil-induced intestinal mucositis by suppression of apoptosis in murine intestinal crypt cells. Br J Pharmacol. 2013;168(6):1388-400.

407. Cheng H, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. III. Entero-endocrine cells. Am J Anat. 1974;141(4):503-19.

408. Hall PA, Coates PJ, Ansari B, Hopwood D. Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. J Cell Sci. 1994;107 (Pt 12):3569-77.

409. Vyas D, Robertson CM, Stromberg PE, Martin JR, Dunne WM, Houchen CW, et al. Epithelial apoptosis in mechanistically distinct methods of injury in the murine small intestine. Histol Histopathol. 2007;22(6):623-30.

410. Knott AW, O'Brien DP, Juno RJ, Zhang Y, Williams JL, Erwin CR, et al. Enterocyte apoptosis after enterectomy in mice is activated independent of the extrinsic death receptor pathway. Am J Physiol Gastrointest Liver Physiol. 2003;285(2):G404-13.

411. Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, Zitvogel L, et al. Cell death modalities: classification and pathophysiological implications. Cell Death Differ. 2007;14(7):1237-43.

412. Vinas JL, Sola A, Hotter G. Mitochondrial NOS upregulation during renal I/R causes apoptosis in a peroxynitrite-dependent manner. Kidney Int. 2006;69(8):1403-9.

413. Denis M. Structure and function of cytochrome-c oxidase. Biochimie. 1986;68(3):459-70.

414. Ghafourifar P, Schenk U, Klein SD, Richter C. Mitochondrial nitric-oxide synthase stimulation causes cytochrome c release from isolated mitochondria. Evidence for intramitochondrial peroxynitrite formation. J Biol Chem. 1999;274(44):31185-8.

415. Zhang J, Jin B, Li L, Block ER, Patel JM. Nitric oxide-induced persistent inhibition and nitrosylation of active site cysteine residues of mitochondrial cytochrome-c oxidase in lung endothelial cells. Am J Physiol Cell Physiol. 2005;288(4):C840-9.

416. Abriata LA, Cassina A, Tortora V, Marin M, Souza JM, Castro L, et al. Nitration of solvent-exposed tyrosine 74 on cytochrome c triggers heme iron-methionine 80 bond disruption. Nuclear magnetic resonance and optical spectroscopy studies. J Biol Chem. 2009;284(1):17-26.

417. Han F, Da T, Riobo NA, Becker LB. Early mitochondrial dysfunction in electron transfer activity and reactive oxygen species generation after cardiac arrest. Crit Care Med. 2008;36(11 Suppl):S447-53.

418. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol. 2013;5(4):a008656.

419. Liu Z, Xi J, Schroder S, Wang W, Xie T, Wang Z, et al. Chimonanthus nitens var. salicifolius Aqueous Extract Protects against 5-Fluorouracil Induced Gastrointestinal Mucositis in a Mouse Model. Evid Based Complement Alternat Med. 2013;2013:789263.

420. Iwanami K, Takeyoshi I, Ohwada S, Kobayashi J, Kawata K, Matsumoto K, et al. Intramucosal pH and intestinal mucosal damage in ischemia-reperfusion injury. Transpl Int. 1998;11(6):401-7.

421. Soldani C, Scovassi AI. Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. Apoptosis. 2002;7(4):321-8.

422. Hengartner MO. The biochemistry of apoptosis. Nature. 2000;407(6805):770-6.

423. Eguchi Y, Shimizu S, Tsujimoto Y. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res. 1997;57(10):1835-40.

424. Petrilli V, Herceg Z, Hassa PO, Patel NS, Di Paola R, Cortes U, et al. Noncleavable poly(ADP-ribose) polymerase-1 regulates the inflammation response in mice. J Clin Invest. 2004;114(8):1072-81.

425. Herceg Z, Wang ZQ. Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. Mutat Res. 2001;477(1-2):97-110.

426. Los M, Mozoluk M, Ferrari D, Stepczynska A, Stroh C, Renz A, et al. Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling. Mol Biol Cell. 2002;13(3):978-88.

427. Hassa PO, Hottiger MO. A role of poly (ADP-ribose) polymerase in NF-kappaB transcriptional activation. Biol Chem. 1999;380(7-8):953-9.

428. Kameoka M, Ota K, Tetsuka T, Tanaka Y, Itaya A, Okamoto T, et al. Evidence for regulation of NF-kappaB by poly(ADP-ribose) polymerase. Biochem J. 2000;346 Pt 3:641-9.

429. Hassa PO, Buerki C, Lombardi C, Imhof R, Hottiger MO. Transcriptional coactivation of nuclear factor-kappaB-dependent gene expression by p300 is regulated by poly(ADP)-ribose polymerase-1. J Biol Chem. 2003;278(46):45145-53.

430. Yung TM, Satoh MS. Functional competition between poly(ADP-ribose) polymerase and its 24-kDa apoptotic fragment in DNA repair and transcription. J Biol Chem. 2001;276(14):11279-86.

431. Bajt ML, Cover C, Lemasters JJ, Jaeschke H. Nuclear translocation of endonuclease G and apoptosis-inducing factor during acetaminophen-induced liver cell injury. Toxicol Sci. 2006;94(1):217-25.

432. Napirei M, Gultekin A, Kloeckl T, Moroy T, Frostegard J, Mannherz HG. Systemic lupus-erythematosus: deoxyribonuclease 1 in necrotic chromatin disposal. Int J Biochem Cell Biol. 2006;38(3):297-306.

433. Ramachandran A, Madesh M, Balasubramanian KA. Apoptosis in the intestinal epithelium: its relevance in normal and pathophysiological conditions. J Gastroenterol Hepatol. 2000;15(2):109-20.

434. Al-Dasooqi N, Bowen JM, Gibson RJ, Logan RM, Stringer AM, Keefe DM. Irinotecan-induced alterations in intestinal cell kinetics and extracellular matrix component expression in the Dark Agouti rat. Int J Exp Pathol. 2011;92(5):357-65.

435. Wu YJ, Muldoon LL, Neuwelt EA. The chemoprotective agent N-acetylcysteine blocks cisplatin-induced apoptosis through caspase signaling pathway. J Pharmacol Exp Ther. 2005;312(2):424-31.

436. Marshman E, Ottewell PD, Potten CS, Watson AJ. Caspase activation during spontaneous and radiation-induced apoptosis in the murine intestine. J Pathol. 2001;195(3):285-92.

437. Labejof LP, Galle P, Mangabeira PA, de Oliveira AH, Severo MI. Histological changes in rat duodenum mucosa after whole-body gamma irradiation. Cell Mol Biol (Noisy-le-grand). 2002;48(5):537-45.

438. Qian W, Nishikawa M, Haque AM, Hirose M, Mashimo M, Sato E, et al. Mitochondrial density determines the cellular sensitivity to cisplatin-induced cell death. Am J Physiol Cell Physiol. 2005;289(6):C1466-75.

439. Letari O, Booth C, Bonazzi A, Garofalo P, Makovec F, Rovati LC, et al. Efficacy of CR3294, a new benzamidine derivative, in the prevention of 5-fluorouracil-induced gastrointestinal mucositis and diarrhea in mice. Cancer Chemother Pharmacol. 2010;66(5):819-27.

440. Li T, Ito K, Sumi S, Fuwa T, Horie T. Protective effect of aged garlic extract (AGE) on the apoptosis of intestinal epithelial cells caused by methotrexate. Cancer Chemother Pharmacol. 2009;63(5):873-80.

441. Sukhotnik I, Shehadeh N, Coran AG, Mogilner JG, Karry R, Shamian B, et al. Oral insulin enhances cell proliferation and decreases enterocyte apoptosis during methotrexate-induced mucositis in the rat. J Pediatr Gastroenterol Nutr. 2008;47(2):115-22.

442. Thorpe D, Stringer A, Butler R. Chemotherapy-induced mucositis: the role of mucin secretion and regulation, and the enteric nervous system. Neurotoxicology. 2013;38:101-5.

443. Laszlo F, Whittle BJ, Moncada S. Time-dependent enhancement or inhibition of endotoxin-induced vascular injury in rat intestine by nitric oxide synthase inhibitors. Br J Pharmacol. 1994;111(4):1309-15.

444. Takizawa Y, Kishimoto H, Kitazato T, Tomita M, Hayashi M. Effects of nitric oxide on mucosal barrier dysfunction during early phase of intestinal ischemia/reperfusion. Eur J Pharm Sci. 2011;42(3):246-52.

445. Stabile AM, Moreto V, Antunes-Rodrigues J, Carnio EC. Central but not systemic inhibition of inducible nitric oxide synthase modulates oxytocin release during endotoxemic shock. Peptides. 2010;31(4):706-11.

446. Babai L, Szigeti Z, Parratt JR, Vegh A. Delayed cardioprotective effects of exercise in dogs are aminoguanidine sensitive: possible involvement of nitric oxide. Clin Sci (Lond). 2002;102(4):435-45.

447. Feinstein-Rotkopf Y, Arama E. Can't live without them, can live with them: roles of caspases during vital cellular processes. Apoptosis. 2009;14(8):980-95.

448. Ostapenko VV, Akagi K, Yamamoto I, Tanaka Y. Indomethacin reduces the skin thermal damage in hyperthermic treatment of experimental malignant tumors. In Vivo. 1999;13(3):255-7.

449. Huang TY, Chu HC, Lin YL, Ho WH, Hou HS, Chao YC, et al. Minocycline attenuates 5-fluorouracil-induced small intestinal mucositis in mouse model. Biochem Biophys Res Commun. 2009;389(4):634-9.

450. Cross RK, Wilson KT. Nitric oxide in inflammatory bowel disease. Inflamm Bowel Dis. 2003;9(3):179-89.

451. Kiang JG, Bowman PD, Lu X, Li Y, Wu BW, Loh HH, et al. Geldanamycin inhibits hemorrhage-induced increases in caspase-3 activity: role of inducible nitric oxide synthase. J Appl Physiol (1985). 2007;103(3):1045-55.

452. Naito Y, Takagi T, Ichikawa H, Tomatsuri N, Kuroda M, Isozaki Y, et al. A novel potent inhibitor of inducible nitric oxide inhibitor, ONO-1714, reduces intestinal ischemia-reperfusion injury in rats. Nitric Oxide. 2004;10(3):170-7.

453. Saleh D, Ernst P, Lim S, Barnes PJ, Giaid A. Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. FASEB J. 1998;12(11):929-37.

454. Clark JA, Lane RH, Maclennan NK, Holubec H, Dvorakova K, Halpern MD, et al. Epidermal growth factor reduces intestinal apoptosis in an experimental model of necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol. 2005;288(4):G755-62.

455. Leong KG, Karsan A. Signaling path ways mediated by tumor necrosis factor alpha. Histol Histopathol. 2000;15(4):1303-25.

456. Takizawa Y, Kitazato T, Ishizaka H, Kamiya N, Tomita M, Hayashi M. Effect of aminoguanidine on ischemia/reperfusion injury in rat small intestine. Biol Pharm Bull. 2011;34(11):1737-43.

457. Sukhotnik I, Pollak Y, Coran AG, Pilatov J, Bejar J, Mogilner JG, et al. Glutamine attenuates the inhibitory effect of methotrexate on TLR signaling during intestinal chemotherapy-induced mucositis in a rat. Nutr Metab (Lond). 2014;11:17.

458. Chen C, Tian L, Zhang M, Sun Q, Zhang X, Li X, et al. Protective effect of amifostine on high-dose methotrexate-induced small intestinal mucositis in mice. Dig Dis Sci. 2013;58(11):3134-43.

459. Ben-Lulu S, Pollak Y, Mogilner J, Bejar J, A GC, Sukhotnik I. Dietary transforming growth factor-beta 2 (TGF-beta2) supplementation reduces methotrexate-induced intestinal mucosal injury in a rat. PLoS One. 2012;7(9):e45221.

460. Koppelmann T, Pollak Y, Mogilner J, Bejar J, Coran AG, Sukhotnik I. Dietary L-arginine supplementation reduces Methotrexate-induced intestinal mucosal injury in rat. BMC Gastroenterol. 2012;12:41.

461. Sukhotnik I, Mogilner JG, Shteinberg D, Karry R, Lurie M, Ure BM, et al. Leptin accelerates enterocyte turnover during methotrexate-induced intestinal mucositis in a rat. Cancer Biol Ther. 2009;8(10):899-906.

462. Horie T, Li T, Ito K, Sumi S, Fuwa T. Aged garlic extract protects against methotrexate-induced apoptotic cell injury of IEC-6 cells. J Nutr. 2006;136(3 Suppl):861S-3S.

463. Sukhotnik I, Mogilner JG, Karry R, Shamian B, Lurie M, Kokhanovsky N, et al. Effect of oral glutamine on enterocyte turnover during methotrexate-induced mucositis in rats. Digestion. 2009;79(1):5-13.

464. Sukhotnik I, Shteinberg D, Ben Lulu S, Bashenko Y, Mogilner JG, Ure BM, et al. Effect of transforming growth factor-alpha on enterocyte apoptosis is correlated with EGF receptor expression along the villus-crypt axis during methotrexate-induced intestinal mucositis in a rat. Apoptosis. 2008;13(11):1344-55.

465. Abd El-Gawad HM, El-Sawalhi MM. Nitric oxide and oxidative stress in brain and heart of normal rats treated with doxorubicin: role of aminoguanidine. J Biochem Mol Toxicol. 2004;18(2):69-77.

466. Hung CR. Role of gastric oxidative stress and nitric oxide in formation of hemorrhagic erosion in rats with ischemic brain. World J Gastroenterol. 2006;12(4):574-81.

467. Ara C, Karabulut AB, Kirimlioglu H, Yilmaz M, Kirimliglu V, Yilmaz S. Protective effect of aminoguanidine against oxidative stress in an experimental peritoneal adhesion model in rats. Cell Biochem Funct. 2006;24(5):443-8.

468. Atasayar S, Gurer-Orhan H, Orhan H, Gurel B, Girgin G, Ozgunes H. Preventive effect of aminoguanidine compared to vitamin E and C on cisplatin-induced nephrotoxicity in rats. Exp Toxicol Pathol. 2009;61(1):23-32.

469. Eroglu C, Yildiz OG, Saraymen R, Soyuer S, Kilic E, Ozcan S. Aminoguanidine ameliorates radiation-induced oxidative lung damage in rats. Clin Invest Med. 2008;31(4):E182-8.

470. Abdel-Zaher AO, Abdel-Rahman MM, Hafez MM, Omran FM. Role of nitric oxide and reduced glutathione in the protective effects of aminoguanidine, gadolinium chloride and oleanolic acid against acetaminophen-induced hepatic and renal damage. Toxicology. 2007;234(1-2):124-34.

CHAPTER – 10

Publications

1. Viswa Kalyan Kolli, Premila Abraham, Bina Isaac, **N. Kasthuri**. Preclinical Efficacy of Melatonin to Reduce Methotrexate-Induced Oxidative Stress and Small Intestinal Damage in Rats. Dig Dis Sci. 2013 Apr;58(4):959-69. **[PMID: 23053903]**

2. Kolli VK, Kanakasabapathy I, Faith M, RamamoorthyH, IsaacB, **Natarajan K**, Abraham P. A preclinical study on the protective effect of melatonin against methotrexate-induced small intestinal damage: effect mediated by attenuation of nitrosative stress, protein tyrosine nitration, and PARP activation. Cancer Chemother Pharmacol. 2013 May;71(5):1209-18. **[PMID: 23420439]**

3. VK Kolli, **K Natarajan**, B Isaac, D Selvakumar, P Abraham. Mitochondrial dysfunction and respiratory chain defects in a rodent model of methotrexate-induced enteritis. Hum Exp Toxicol. 2014 Oct;33(10):1051-65. **[PMID: 24347301]**