RESULTS, ANALYSIS AND DISCUSSION
STUDY I

Light microscopic structural changes in the small intestine of rat after treatment with methotrexate.
Abstract

MTX is widely used in the treatment of malignancies, psoriasis, rheumatoid arthritis, ectopic pregnancy and eczema. The toxic side effects limit the usage of the drug. Previous studies have reported the small intestine damage following MTX treatment such as diarrhea, villus atrophy, impaired digestive and absorptive function, loss of barrier function, and damage to the mitochondria in the epithelial cells. The purpose of this study was to study and characterize MTX induced damage in the duodenum, jejunum and ileum regions of the small intestine. To study this, the rats were treated with MTX with 7mg/kg body weight intraperitoneally for three consecutive days. 12 hours and 24 hours after the final dose the rats were euthanized, the entire small intestine was taken, washed with ice cold saline, weighed and used for histological studies by light microscopy. The rats had diarrhea and weight loss after the administration of MTX. The weight of the intestine was also decreased in response to MTX. Light microscopic examination of the small intestines revealed damage to the duodenum, ileum and jejunum. Treatment of rats with MTX damaged the villi and crypt cells. The villi were atrophied, blunted and focally absent. The crypts of Lieberkuhn were found to be less in number and were accompanied by the presence of crypt abscess. The villus/crypt ratio was decreased and the overall thickness of the mucosa was decreased accompanied by transmural inflammatory infiltrate. In conclusion, MTX at a dose of 7 mg/kg body weight given intraperitoneally for three consecutive days had caused architectural damage in the duodenum, jejunum and ileal regions of the small intestine, the damage being more severe at 24 hours after the final dose of MTX.
### 6.1.1. Introduction

Gastrointestinal (GI) toxicity is of major clinical concern and is often a major cause of cancer treatment-related morbidity (106). Even though there are some preliminary clinical studies and reports, there does not appear to be an extensive examination of gastrointestinal toxicity. It has been shown that chemotherapy alters mucosal morphology and gut barrier function (343). Previous studies have reported histological abnormalities in the small intestine following methotrexate therapy in children with acute lymphoblastic leukemia (ALL) (19, 344). MTX is shown to cause severe jejunal injury, diarrhea, weight loss and damage to villus architecture. MTX was known to increase intestinal permeability by the paracellular pathway, which may affect the absorption of other molecules (345). Cancer chemotherapy has to be accompanied by symptomatic therapy such as antibiotics and anti-diarrheal drugs. MTX has also been shown to deteriorate the barrier function of mucosa against intravascular bacteria (21) and alter the absorptive function (23), and intestinal permeability (346). Mitochondrial damage in the epithelial cells and oxidative damage to the mitochondrial lipids and DNA were also reported in response to MTX (113).

MTX has been used to establish in rodents a reproducible model of small intestinal enteropathy characterized by diarrhea, villus atrophy and impaired digestive and absorptive function (320, 347). Different doses of MTX were used to study the small intestine toxicity of MTX ranging from 1.25 mg/kg body weight for 3 consecutive days to a single dose of 500 mg/kg body weight through intraperitoneal, intravenous and subcutaneous routes. In this study, a dose of 7mg/kg body weight for 3 consecutive days by intraperitoneal route was chosen for the rat model of MTX – induced small intestine damage as suggested by Warden et al (24). The previous studies that had reported small intestine changes in response to MTX were done mostly on the jejunum samples (20, 24, 347-358). Little is known about the effect of MTX on the whole small intestine at a dose of 7mg/kg body weight.
of MTX given intraperitoneally for 3 consecutive days. This study was designed to characterize the methotrexate–induced morphological changes in the duodenum, jejunum and ileum regions of the small intestine in rat model.

6.1.2. Summary of experiment

A pilot study was carried out in order to determine the optimum dose of MTX that is required to produce gastrointestinal damage with minimum mortality of rats. MTX was administered intraperitoneally at the dose of 25 mg/kg body weight. This dose is almost equivalent to therapeutic dose of 1 g/m² in humans (359) and has been used earlier by other workers in order to establish a rat model of MTX induced enteropathy (360, 361). Eighty percent (8/10) of the rats that received a single dose of 25 mg/kg body weight died within 12 h of treatment. Therefore, we chose a lower dose of MTX as recommended by Warden et al. (24). After the intraperitoneal administration of methotrexate at the dose of 7 mg/kg body weight for three consecutive days, the rats presented with histological evidence of drug–induced small intestinal enteropathy and villus atrophy similar to that seen in humans. Besides, all the rats survived the treatment period of 3 days. Therefore the dose of 7 mg/kg body weight was used in the study. The rats were divided into three groups and treated as follows:

Group 1: The rats in this group (n = 6) received the vehicle (NaCl–NaOH; pH 7.4) alone for 3 days.

Group 2: The rats in this group (n = 8) received three consecutive daily intraperitoneal injections of methotrexate at the dose 7 mg/kg body weight and were sacrificed 12 hours after the final dose of MTX.
Group 3: The rats in this group (n = 8) received three consecutive daily intraperitoneal injections of methotrexate at the dose 7 mg/kg body weight and were sacrificed 24 hours after the final dose of MTX.

After overnight fasting, the rats were sacrificed and the entire length of small intestines were removed, flushed with cold PBS, weighed and divided into duodenum, jejunum and ileum. The sections were fixed in 10% buffered formalin then embedded in paraffin, sectioned and stained with haematoxylin and eosin for examination by light microscopy.

6.1.3. Mucosal injury gradation

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. (362). Briefly, the mucosal damage was graded from 1 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.

6.1.4. Results

The methotrexate treated rats had lost weight when compared with the control. The weight loss was 6%. Besides, the MTX treated rats had diarrhea. The weights of the intestine were significantly reduced (by 38%) in the MTX treated rats as compared with controls (2.00 ± 0.30 vs 1.25 ± 0.15 g/100 g body wt, P < 0.01).
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.

Twelve hours after the final dose of MTX treatment, the villi were found to be blunted and there was mild destruction of the crypts of lieberkuhn in the duodenum (Figure: 6.1.2 A). In the jejunum, there was distortion of the villi with mild blunting and the crypts of lieberkuhn were decreased in number. The crypts of lieberkuhn were destroyed and the crypt abscesses were present (Figure: 6.1.2 B). In the ileum, the villi appeared normal, but the crypts of lieberkuhn were reduced in number (Figure 6.1.2 C). There was prominent transmural inflammatory infiltrate that was confined to the mucosal layer.

Twenty four hours after the final dose of MTX, there was moderate destruction of the villi and the crypts of lieberkuhn in duodenum (Figure: 6.1. 3 A). In jejunum, there was more destruction of the villi. The villi were atrophied and focally absent. The crypts of lieberkuhn were very few in number as compared with 12 hour group (Figure: 6.1.3 B). In ileum, the villi were atrophied, blunted and fused. There was destruction of the crypts of lieberkuhn (Figure: 6.1.3 C). Twenty four hours after the final dose of MTX, 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 three groups were graded on a 5 point scale. The small intestine sections in group I revealed normal morphology with normal villi and crypt cells (Grade 0). The duodenum and jejunum sections in group II (12 hours) revealed blunted and denuded villi along with the extension of the sub epithelial space with the epithelial layer lifting up in sheets (Grade 3). The ileum sections in group II (12 hours) revealed development of sub epithelial Gruenhagen’s space at the apex of the villus and reduced number of crypt cells (Grade 1 & 2). The duodenum and jejunum sections in group III (24 hours) showed atrophied villi with exposed lamina propria and there was acute inflammatory infiltrate (Grade 4). The ileum sections from the group III (24 hours) revealed fused villi, digestion and disintegration of the lamina propria in villi and presence of hemorrhage and ulceration (Grade 5).

The damage comparatively was more in the duodenum and jejunum (Grade 3) followed by ileum (Grade 2) twelve hours after the final dose of MTX. The damage was comparatively more in the ileum (Grade 5), followed by jejunum and duodenum (Grade 4) twenty four hours after the final dose of MTX.
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 Leibergkun; ME – Muscularis Externa
Figure: 6.1.2

Histology of small intestine 12 hours after the final dose of MTX, magnification X100 (n = 8)

(A) Duodenum showed slight blunting and shortening (black arrow) and there was destruction of the crypts of leiberkuhn.

(B) Jejunum showed degeneration of surface epithelium and crypt abscess (white arrow) is observed with inflammatory infiltrate

(C) Ileum showed normal villi, but there was damage to the crypts of leiberkuhn.

Black arrows indicate villus shortening, blunting and degeneration.

White arrows indicate the crypt abscess
Figure: 6.1.3

Histology of small intestine 24 hours after the final dose of MTX, magnification X100. (n = 8). Black arrow indicates the 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).
6.1.5. Discussion

MTX therapy is often accompanied by side effects such as nausea, vomiting, diarrhea, gastrointestinal ulceration and mucositis (23, 305). The damage to the rat small intestine mirrors that in the human (363). MTX is also known to cause malabsorption syndrome characterized by decrease in the surface area for absorption occurring due to histological changes such as shortened villi, resulting in disrupted intestinal permeability causing a lesser absorption of nutrients (20, 23, 357, 364-366). Majority of earlier studies showing MTX induced small intestinal damage were done in the jejunum. In this study, morphological study was done in the duodenum, jejunum and ileum sections to study the MTX – induced small intestinal damage with a dose of 7 mg/ kg body weight of MTX given intraperitoneally for three consecutive days.

The results of this study showed that MTX causes architectural damage to the small intestine and is characterized by villus distortion, fusion and blunting, epithelial atrophy, and loss of crypt cells. There was also an inflammatory response as evidenced by acute transmural inflammatory infiltrate, and goblet cell depletion in small intestinal tissue. These findings are in agreement with the earlier studies (20, 353, 358, 367,368). The above mentioned features in the small intestine of rat resembles to that of gut mucositis experienced as a common side – effect by patients undergoing chemotherapy or radiotherapy (111). These characteristics of mucositis were also observed in other studies (20, 369-371). Apoptosis of crypt cells is suggested to play a role in the increased permeability and villus atrophy (372-374).

Earlier studies have shown a decrease in the villus height and crypt depth, decrease in the frequency and length of crypts and damage of the mucosa of the small intestine in response to MTX (23, 346, 366, 368, 375-377). Thus MTX treatment damages the small intestine
morphologically, and is accompanied by a decrease in the constituents of the small intestinal mucosa (304,305). MTX was also shown to decrease epithelial cell turnover, bowel and mucosal weight resulting in mucosal hypoplasia of the small intestine. Mucosal hypoplasia was thought to be a result of both hypo proliferation and widespread apoptosis of stem cells in intestinal crypts secondary to chemotherapy, which lead to villus atrophy and absorptive dysfunction (378). Nakamaru et al (379) have shown that the permeation of non absorbable markers through the small intestine of mice is enhanced by MTX treatment in contrary to previously reported results indicating that MTX treatment decreases both active and passive transport in the small intestine (364, 380-385).

Proliferation of small intestinal epithelial cells occurs in the crypt. The crypt cells, which rapidly generate, migrate to the villus tip, and replacement of the intestinal epithelium is complete in about 3 days in humans (386) and mice (387), and in about 2 days in rats (388). The damage to the crypt cells and the accompanied inflammatory response give rise to the formation of crypt abscess in response to MTX (21, 24, 102, 306, 315, 318, 348, 353, 366, 368, 389). The MTX – induced morphologic changes in the small intestine is thought to be triggered by crypt cell damage (303). Villus atrophy and inflammatory infiltrate observed in this study in response to MTX may be a consequence of the effect of the drug on the proliferating crypt cells.

In conclusion, MTX caused acute inflammatory changes accompanied by damage to the villi and crypts of lieberkhun in the various segments of small intestine. The damage was more in the ileum and jejunum. Villus epithelial cells are involved in nutrient absorption and these cells are atrophied in the MTX treated rats. This results in decreased nutrient absorption by the small intestine. This explains the cause of diarrhea and weight loss seen in MTX treated rats.
STUDY II

Role of reactive oxygen species and neutrophil infiltration in methotrexate induced small intestinal damage.
Abstract

Oxidative damage of small intestine has been reported by previous studies in response to MTX, but the mechanism by which MTX causes damage has not been completely characterized. In this study, it is hypothesized that MTX administration causes increased generation of reactive oxygen species (ROS) and oxidative damage in the small intestine. Adult male Wistar rats were treated with 7 mg / kg body weight of MTX intraperitoneally for 3 consecutive days and the rats were sacrificed 12 hours and 24 hours after the final dose of MTX. Vehicle treated rats served as control. The intestinal tissue was used for various biochemical studies. The markers of oxidative damage including malondialdehyde, conjugated dienes, protein carbonyl content, protein thiol, and reduced glutathione were assayed. The activities of the glutathione (major antioxidant) requiring enzymes, namely glutathione peroxidase, glutathione S transferase, glutathione reductase and myeloperoxidase activity were also assayed in the intestinal homogenates along with other antioxidant enzymes including catalase, superoxide dismutase. The markers of oxidative stress such as malondialdehyde, conjugated dienes, protein carbonyl content, and the activity of glutathione peroxidase was significantly increased in the intestines of MTX treated rats. There was marked neutrophil infiltration as evidenced by increase in myeloperoxidase activity in the small intestines after treatment with methotrexate. Reduced glutathione and the activities of glutathione reductase, superoxide dismutase and catalase were significantly decreased in the intestines of MTX treated rats as compared to control group. In plasma, the levels of MDA and protein carbonyl were increased significantly after administration of MTX as compared to control group.

In conclusion, this study showed that MTX induces oxidative stress in the small intestine. The activities of the antioxidant enzymes were altered; especially those involving glutathione
suggesting that the ROS – induced decrease in the activities of the antioxidant enzymes may contribute to MTX induced small intestinal damage. The infiltration of activated neutrophils play an important role in the MTX – induced increased ROS production. Thus, the increased ROS production and hence oxidative stress in response to MTX may due to activated neutrophils or altered antioxidant enzymes or damaged mitochondria or by direct effect of MTX.
6.2.1. Introduction

MTX is an anti-folate drug widely used in the treatment of malignancies. However, the efficacy of this drug is often limited by severe side effects especially on the rapidly dividing cells of bone marrow and the gut mucosa. Though some mechanisms have been suggested to play a role in MTX induced small intestinal damage, the mechanism of gastrointestinal toxicity of methotrexate has not been clarified completely.

One of the major side effects of MTX is mucositis characterized by crypt loss, villus fusion and atrophy patients undergoing chemotherapy or radiotherapy (20, 111). The small intestinal damage induced by MTX treatment results in malabsorption and diarrhea (18). This malabsorption results in weight loss and disturbs the cancer chemotherapy of patients. It has been proposed that inflammation of gut mucosa causes impairment of antioxidant defense mechanism and makes tissues more susceptible to oxidative stress (390). MTX induced small intestinal damage was characterized by marked inflammation (2, 111). Increased production of reactive oxygen species and oxidative stress has been postulated for such damage (21, 107).

Reactive oxygen species (ROS) have been reported to initiate irreversible oxidative damage in the cells increasing membrane lipid peroxidation; protein oxidation and DNA damage (391). MTX is known to inhibit cytosolic NAD(P) – dependent dehydrogenases (38, 392) and NADP malic enzyme thereby decreasing the availability of NADPH, thus interfering with HMP shunt (39) and nucleic acid metabolism. Previous study had shown that MTX caused decreased activity of the succinate dehydrogenase complex of the electron transport chain. Since, mitochondria are considered as the major source of ROS, MTX might act through the mitochondrial ROS production to cause small intestinal damage. In this study it was hypothesized that MTX
administration causes increased ROS generation and oxidative damage that alters the antioxidant defense system in the small intestine.

**6.2.2. Summary of experiment**

Adult male Wistar rats (200 – 250g) were treated with 7mg/ kg body weight of MTX intraperitoneally for three consecutive days. Control animals received an equal volume of the vehicle for the drug. The rats were sacrificed 12 hours and 24 hours after the final dose of methotrexate. The rats were weighed anesthetized with halothane and blood obtained by cardiac puncture. The entire length of small intestine was removed, washed and the mucosa was scraped off from the small intestine using glass slide. The mucosa was weighed and homogenized in appropriate buffers for the biochemical assays.

**6.2.3. Results**

i) **Effect of MTX on oxidative stress parameters and neutrophil infiltration**

MTX treatment had significantly increased the levels of lipid peroxidation markers – malondialdehyde (MDA) and conjugated diene in the 12 hour and 24 hour groups as compared to controls. MDA levels were increased by 100% at 12 hours and by 115% at 24 hours after the final dose of MTX (Figure. 6.2.1), and the Conjugated dienes were increased by 3.5 fold at 24 hours after treatment with MTX (Figure. 6.2.2). Protein carbonyl content, a sensitive, reliable and early marker of oxidative damage to proteins was increased by 4 fold at 12 hours and by 2.5 fold at 24 hours after treatment with MTX as compared to controls (Figure. 6.2.3). Protein thiol, an important free radical scavenger was decreased by 38% 24 hours in response to MTX as compared to controls (Figure. 6.2.4). Reduced glutathione was decreased by 54% at 12 hours and by 39% at 24 hours after MTX treatment as compared to controls (Figure. 6.2.5). Myeloperoxidase (MPO)
activity that indicates neutrophil infiltration activity was increased by 3 fold at 12 hours and nearly 4 fold at 24 hours after the final dose of MTX as compared to controls (Figure. 6.2.6).

ii) Effect of MTX on the activities of antioxidant enzymes

Significant changes were seen in the activities of antioxidant enzymes following methotrexate treatment. Glutathione peroxidase activity (GPO) was increased 4 fold at 12 hours and 2.5 fold at 24 hours after treatment with MTX as compared to controls (Figure. 6.2.7). The activity of Glutathione S transferase (GSTase) was not altered significantly by MTX as compared to controls (Figure. 6.2.8). However, the activity of glutathione reductase, an enzyme involved in the regeneration of reduced glutathione was decreased at 24 hours as compared to controls (Figure. 6.2.9). The activities of the anti-oxidant enzymes, catalase (Figure. 6.2.10) and superoxide dismutase (Figure. 6.2.11) were decreased significantly 12 hours and 24 hours after the final dose of MTX as compared to controls.

The oxidative stress observed in the small intestine was also reflected in the plasma levels of MDA and protein carbonyl that have increased significantly as compared to controls. In plasma, the levels of MDA (Figure. 6.2.12) and protein carbonyl (Figure. 6.2.13) were significantly increased at 12 hours and 24 hours after the final dose of MTX as compared to controls. There was no significant difference in the thiol groups (Figure. 6.2.14) and GSTase activity (Figure. 6.2.15) between MTX treated rats and control rats.
Malondialdehyde levels in the small intestines of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n = 6 (control group); n = 8 (MTX group), * P < 0.01 as compared to control.

Conjugated diene levels in the small intestines of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n = 6 (control group); n = 8 (MTX group), * P < 0.01 as compared to control. # P < 0.01 as compared to 12 hours.
Figure: 6.2.3

Protein carbonyl content in the small intestines of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group), * P < 0.02, ** P < 0.01 as compared to control.

Figure: 6.2.4

Protein thiol levels in the small intestines of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group).
Reduced glutathione levels in the small intestines of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group), * P < 0.05 as compared to control.

Myeloperoxidase activity in the small intestines of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group), * P < 0.05, ** P < 0.01 as compared to control.
Glutathione peroxidase activity in the small intestines of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group), * P < 0.01 as compared to control.

Glutathione S transferase activity in the small intestines of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group).
Glutathione reductase activity in the small intestines of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n = 6 (control group); n = 8 (MTX group), * P < 0.02 as compared to control. # P < 0.01 as compared to 12 hours.

Catalase activity in the small intestines of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n = 6 (control group); n = 8 (MTX group), * P < 0.05, ** P < 0.01 as compared to control.
Superoxide dismutase activity in the small intestines of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n = 6 (control group); n = 8 (MTX group), * P < 0.05 as compared to control.

Malondialdehyde levels in the plasma of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n = 6 (control group); n = 8 (MTX group), * P < 0.05 as compared to control.
Protein carbonyl levels in the plasma of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n = 6 (control group); n = 8 (MTX group), * P < 0.05, ** P < 0.01 as compared to control.

Protein thiol levels in the plasma of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n = 6 (control group); n = 8 (MTX group).
Glutathione S transferase activity in the plasma of control rats and experimental rats 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group).
6.2.4. Discussion

Methotrexate treatment is associated with severe side effects. MTX nonspecifically acts on both malignant and normal cells (347). MTX is known to cause small intestinal damage such as mucositis and enterocolitis that may limit the usage of the drug in chemotherapy regimens (118, 389, 393). Different mechanisms were suggested to play in MTX – induced small intestinal damage (21, 25, 107). The reason for side effects were thought to be allergic, cytotoxic, immunological and oxidative (107, 394). The intestinal barrier dysfunction seen in the MTX – treated rats was thought to be ROS induced dephosphorylation and altered localization of zonula occludens 1 (ZO-1) that may lead to tight junction (TJ) barrier disturbances and thus the enhanced intestinal permeability (395). Oxidative stress was suggested as the possible mechanism in MTX- induced central nervous system injury in children undergoing chemotherapy for ALL (396, 397); in MTX- induced liver damage (398-402) and MTX- induced renal damage (35, 403, 404). In this study, it is hypothesized that MTX administration causes increased ROS generation and oxidative damage in the small intestine.

Reactive oxygen species (ROS) reacts with polyunsaturated fatty acids producing lipid peroxides. Lipid peroxidation was known to cause cellular membrane damage. The results of this study showed increased levels of MDA and conjugated diene indicating the importance of ROS – induced lipid peroxidation in MTX – induced small intestinal toxicity. The results of this study are in agreement with the previous studies on small intestine that showed increased lipid peroxidation by MTX (107, 316, 318, 394). Recent studies have also reported increased MTX – induced lipid peroxidation in the small intestine (314, 315, 389, 405). The protein carbonyl content is reported to be a sensitive and early marker of oxidative stress to tissues as compared
with lipid peroxidation (328). The present study showed for the first time an increase in protein carbonyl content in the intestines following treatment with MTX at both the time points. It is well documented that protein oxidation marks the protein for degradation (406). Proteins (enzymes) regulate various metabolic pathways and damage to proteins may result in the alteration of normal metabolic pathways resulting in cell death and tissue damage. The tissue changes in the levels of MDA and protein carbonyl were also reflected in plasma levels. In contrast to our findings, Bauerova K et al had shown that MTX had decreased the plasma levels of MDA and protein carbonyl in adjuvant arthritis rat model (407). The authors attribute this to the anti-inflammatory and immunosuppressive effects of MTX. Reactive oxygen species (ROS) produced by methotrexate are suggested to be highly reactive causing small intestinal damage by reacting with PUFAs of membranes, nucleotides of DNA and sulphydryls of proteins (389).

Reactive oxygen species are known to trigger the accumulation of leucocytes in the tissues involved, and thus aggravate tissue injury indirectly through activated neutrophils. The formation of ROS and neutrophil accumulation disrupts the microcirculation of the intestine mucosa and plays a key role in the formation of ulcer (21). The activated neutrophils secrete myeloperoxidase and other proteases leading to more free radical production (107, 394). Myeloperoxidase is known to play an important role in the production of oxidants by neutrophils. In this study, a marked elevation in MPO activity was observed at both the time points in response to MTX treatment, indicating that neutrophil infiltration might contribute to MTX induced oxidative stress and small intestinal damage. Activated neutrophils are not the only sources of ROS in MTX induced small intestinal damage. Miyazono and Horie (107) have demonstrated that xanthine oxidase is an important source of ROS in MTX induced small intestinal damage, in addition to myeloperoxidase.
Reduced glutathione is the most abundant non-protein molecule in the cell and plays an important role in maintaining the balance between oxidation and antioxidation. A previous study has shown that depletion of reduced glutathione in tissues promotes oxidative stress and tissue injury (408). In the current study, the level of reduced glutathione was decreased in the small intestine of rat after treatment with MTX. Depletion of reduced glutathione may be a consequence of and/ contribute to MTX induced oxidative stress. The increase in the activity of the free radical detoxifying enzyme, glutathione peroxidase in the small intestines of MTX treated rats observed in the present study may be an adaptive mechanism by the cells to detoxify the reactive oxygen species in order to minimize tissue damage. The increased utilization of the reduced glutathione by these enzymes may account for the decrease in the reduced glutathione content in the small intestine. The activity of glutathione reductase, the enzyme crucial for the regeneration of reduced glutathione was increased initially but 24 hr after treatment with MTX the activity was significantly less than that of control. The reduced activity of this enzyme may account for the decreased availability of reduced glutathione for scavenging reactive oxygen species, thereby rendering the epithelial cells susceptible to increased oxidative stress and tissue injury. NADPH is utilized by glutathione reductase to maintain the reduced state of cellular glutathione (318). It has been shown earlier that the cytosolic NADP – dependent dehydrogenases (38, 392) and malic enzyme (39) are inhibited by MTX, indicating that the drug could decrease the availability of NADPH by inhibiting the pentose cycle enzymes (39). The reduced availability of NADPH may be responsible for the decreased activity of glutathione reductase and hence reduced level of reduced glutathione observed in the present study. Thus significant decrease in reduced glutathione levels promoted by MTX, leads to a reduction of effectiveness of the antioxidant enzyme defense system, thereby sensitizing the cells to reactive oxygen species (392). The activities of the antioxidant enzymes – catalase and superoxide
dismutase were found to be decreased in the small intestines of MTX treated rats suggesting the utilization of these enzymes for quenching the free radicals. In contrast to our study, Cirakil et al had shown an increase in reduced glutathione levels and catalase and superoxide dismutase activities in the small intestine after treatment with methotrexate (318). These changes were thought to be because of up regulation of these proteins in the small intestine in response to MTX induced oxidative stress. The results of our study show evidence for the role of oxidative stress in MTX induced small intestinal damage. Studies reported earlier and those published subsequently support our findings. Antioxidants such as N acetyl cysteine and carotene have been shown to prevent MTX induced oxidative stress and small intestinal damage (316, 318). Gulgun et al have demonstrated that proanthocyanidin prevents MTX induced oxidative stress and small intestinal damage (389). Maeda et al have demonstrated that oxidative stress causes enhanced permeability in the small intestines of MTX treated rats (405). Very recently, Somi et al (409) showed lipoic acid protects against MTX induced oxidative stress and small intestinal damage in a rabbit model.

In summary, this study reveals that MTX induces oxidative stress in the small intestine. MTX induced oxidative stress may be due to overproduction of ROS (probably by activated neutrophils) and or depletion of the antioxidant enzymes; especially those involving glutathione. It is therefore suggested that oxidative stress, neutrophil infiltration and altered activities of antioxidant enzymes contribute to MTX induced small intestinal damage.
STUDY III

Role of reactive nitrogen species in methotrexate induced small intestinal damage.
Abstract

MTX is commonly used in the treatment of different malignancies and inflammatory conditions. Gastrointestinal toxicity is one of the most serious side effects in the methotrexate (MTX) treatment. However, the mechanism by which MTX causes toxicity has not been completely clarified, which may be the reason why symptomatic therapy is carried out. Our previous studies have showed the involvement of oxidative stress in the pathogenesis of MTX – induced small intestinal damage. On the other hand, nitric oxide and its metabolite peroxynitrite were suggested to have important role in gut barrier failure, inflammation and epithelial injury in the small intestine. In the current study, the focus was on the nitrosative stress in order to clarify the mechanism of MTX – induced small intestinal damage. Methotrexate (7mg/kg body weight) was administered intraperitoneally to rats for three consecutive days. The rats were sacrificed 24 hours after the final dose of methotrexate. The small intestinal segments were fixed for immunohistochemical localization of nitrotyrosine and PARP. Mucosal homogenates were prepared from other intestinal segments and used for the assay of nitrite levels, by which nitric oxide production was evaluated. MTX caused increased NO production as revealed by significant increase in the tissue nitrate levels as compared to control. The staining for nitrotyrosine was also found to be more in MTX treated rats as compared to control indicating the formation of peroxynitrite, a potent free radical. The immuno – expression of PARP was increased in response to MTX as compared to control, suggesting the important role of PARP in the pathogenesis of MTX in small intestine. The nitrotyrosine staining was confined to the mucosa, especially lamina propria. PARP expression was seen in both the mucosa and the muscularis with the expression being more in the tips of the villus. In conclusion, the present study demonstrates the involvement of increased nitric oxide production and PARP in MTX – induced small intestinal damage in rat.
6.3.1. Introduction

Intestinal epithelium is a highly proliferative organ in which stem cell positions are defined in terms of the spatial arrangement within the crypt (410). 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, resulting in replacement of lining cells every two to three days (411). Apoptotic cells are normally observed at the tips of villi as well as in crypts (411).

Methotrexate administration is known to cause ulceration, mucosal damage, malabsorption, diarrhea (2, 111) and was also accompanied with diminished barrier function and permeability of mucosa leading to invasion of gut flora into the circulation (18). Overproduction of nitric oxide and its metabolite peroxynitrite are suggested to play an important role in gut barrier failure and inflammation (412).

Constitutive expression of iNOS has been described in normal intestinal epithelium in several species including mice and guinea pigs (413-416). The iNOS activity of epithelium is capable of nanomolar conversion of arginine to NO (413, 417) and the constitutive isoforms of NOS produce picomolar amounts of NO, and are associated with preservation of the mucosal barrier (418-420). This accounts for the basal staining for nitrotyrosine in the small intestines of control rats. In models of chronic intestinal inflammation, increased NO production has been demonstrated to exacerbate injury by combining with superoxide to generate more potent free radicals such as peroxynitrite (415).

Since peroxynitrite is a transient species with a biological half-life even shorter than that of nitric oxide (421), it immediately nitrates specific tyrosine residues on proteins (422). Therefore nitrotyrosine is used as an indirect indicator of peroxynitrite content and hence NOS activity (161,
The increased vulnerability of villus epithelium to injury coupled with an ability to constitutively and rapidly up regulate NO synthesis suggests the intriguing possibility that iNOS plays an important role in acute epithelial defense or repair (424).

Increased immunohistochemical staining of nitrotyrosine has been observed in inflammatory conditions such as inflammatory bowel disease and ulcerative colitis (425-427). Increased production of NO, extensive apoptosis and 3-nitrotyrosine staining were reported earlier in necrotizing enterocolitis in both humans and rodents (428). Up regulation of iNOS and 3-nitrotyrosine labeling were seen in the intestinal mucosa of patients with active ulcerative colitis and the deleterious effects of sustained NO production in gut inflammation have also been shown in humans (425, 429-432). Localized production of nitric oxide in villus enterocytes was shown in inflammatory condition resulting in gut barrier failure and an increase in enterocyte apoptosis (433). MTX-induced NO production was reported earlier in hamster oral mucositis (434).

PARP (mainly PARP – 1) is a nuclear nick sensor, protein modifying and nucleotide-polymerizing enzyme, which is abundantly present in the nucleus. PARP – 1 acts as a survival factor for intestinal stem cells in vivo at low DNA damage levels. In stark contrast, there is a risk of necrotic cell death due to severe NAD$^+$ and ATP depletion when PARP – 1 is acutely over-activated (435).

Previous studies demonstrated that intestinal epithelial cells (IEC) constitutively contain the machinery of cell death, which is rapidly activated on loss of anchorage, leading to caspase activation within minutes of detachment leading to DNA fragmentation in less than 2 hours (436). PARP activation can be triggered by a variety of environmental stimuli and free radical/oxidants, most notably hydroxyl radical and peroxynitrite (210). The induction of DNA single strand breaks by hydroxyl radical (437) and by peroxynitrite (438-442) is well known. It has been shown that whole body irradiation results in death of PARP-1 knockout (PARP$^{-/-}$) mice within a few days, due
to severe disruption of intestinal epithelial lining as compared to wild type mice (443). MTX was shown to cause apoptosis in proliferating CD4\(^+\) T cells by Poly (ADP-ribose) polymerase (PARP) cleavage (444). The present study was aimed to investigate the role of nitric oxide and PARP in MTX induced small intestinal damage.

6.3.2. Summary of experiment

Adult male Wistar rats weighing 200 – 220g were used. The rats were administered with MTX (7 mg/kg body weight) intraperitoneally for three consecutive days. The rats were divided into two groups and treated as follows:

Group 1: The rats in this group (n = 8) received three consecutive daily intraperitoneal injections of methotrexate at the dose 7 mg/kg body weight dissolved in NaCl – NaOH (pH 7.4). Group 2: The rats in this group (n = 6) received the vehicle alone for 3 days.

The animals were sacrificed 24 hours after the final dose of MTX after overnight fast and the entire length of small intestine was removed. The intestine was then washed with cold saline and the mucosa was then scraped using a glass slide. The scrapings were homogenized in appropriate buffer and assayed for nitrate levels. Duodenum, jejunum and ileum samples of the small intestine were taken from MTX group as well as control group for the immunolocalization of nitrotyrosine and PARP in paraffin embedded tissue using HRP as a secondary marker which is demonstrated using DAB as a chromogen. The epithelium of villi and connective cells of the lamina propria were examined for the presence of apoptotic cells and necrotic cells. Apoptotic cells were identified as small cells (smaller than normal) with homogeneous (relatively dark stained) nuclei and clear cytoplasm in immunostaining sections for PARP expression. Necrosis was assessed by looking for the presence of i) vascular congestion; ii) altered villi & crypt shape; iii) blunting, bloating &
fusion of adjacent villi; iv) increased cellularity in lamina propria; v) edema & vi) desquamation of cells.

6.3.3. Results

The nitrate level in the small intestine tissue was increased fourfold as compared with control and 24 h after the final dose of MTX. The elevated tissue levels of nitrate were also reflected in the plasma (Figure. 6.3.1).

In the control group, nitrotyrosine expression was present in the apical region of the villi epithelial cells as well in glandular epithelium, in the connective tissue and in the muscle tissue of the small intestine. However, there was variation in the intensity of expression with the duodenum (Figure: 6.3.2 A) showing a higher level of nitrotyrosine followed by jejunum (Figure: 6.3.2 B) and ileum (Figure: 6.3.2 C).

In the MTX group, destruction of mucosal integrity and vasodilatation were the unique features observed. Intense patches of brown reaction product, indicating elevated level of nitrotyrosine, were seen in the villi epithelium where the cells as such could not be delineated from the surrounding connective tissue. Connective tissue cells’ infiltration of the lamina propria and relatively higher level of nitrotyrosine in these cells were noted. In the duodenum, the enterocytes lining the entire villi and the crypts of lieberkuhn showed high levels of nitrotyrosine staining (Figure: 6.3.2 D). In jejunum, the enterocytes lining the villi, intestinal glands (crypts of Lieberkuhn) and smooth muscle fibers had high levels of nitrotyrosine staining (Figure: 6.3.2 E). In ileum, the enterocytes lining the villi, intestinal glands, cells in lamina propria, payers’ patch and smooth muscle fibers were strongly stained for nitrotyrosine (Figure: 6.3.2 F). The severity of occurrence of nitrotyrosine & morphological changes were high in ileum (Figure: 6.3.2 F) with almost equal level of nitrotyrosine presence in duodenum (Figure: 6.3.2 D) and jejunum (Figure: 6.3.2 E).
Nitrate levels in the small intestines and plasma of control rats and experimental rats at 12 hours and 24 hours following treatment with MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group), * P < 0.01 as compared with control.
Nitrotyrosine levels in rat small intestine 24 hours after treatment with MTX evaluated by immunohistochemistry. 40X

(A – C) Low levels of nitrotyrosine in the apical region of the villi epithelial cells as well in glandular epithelium, in the connective tissue and in muscle tissue of duodenum (A), jejunum (B) and ileum (C).

(D – F) Elevated levels of nitrotyrosine in the villi epithelium of duodenum (D), jejunum (E) and high levels of nitrotyrosine in the ileum (F).
Immunolocalization of PARP revealed that the expression of PARP in duodenum, jejunum and ileum was more in response to MTX as compared to control group. In control group, there was uniform and moderate distribution of PARP in villi, crypts and the smooth muscle fibers (Figure: 6.3.3 A – C). In the MTX treated group, the expression of PARP was more in ileum followed by duodenum and jejunum. In duodenum, the PARP expression was seen in the villi and crypts with the focal expression of high intensity focal points (Figure: 6.3.3 D). In jejunum, there was occasional focal expression of high intensity PARP expression (Figure: 6.3.3 E). In ileum, the PARP expression was seen more in the villi and crypts with high intensity focal points (Figure: 6.3.3 F).
Expression of PARP in the small intestine of rat 24 hours after treatment with MTX by immunohistochemistry. 40X

(A – C) Minimal expression of PARP in the apical region of the villi and crypts in duodenum (A), jejunum (B) and ileum (C).

(D – F) Increased expression of PARP in the apical region of the villi and crypts in duodenum (D), jejunum (E) and ileum (F).
The number of apoptotic cells in the villi epithelium of MTX treated rats was not different from that of controls, suggesting that MTX treatment does not trigger apoptosis (programmed cell death) in the villi epithelium of small intestine.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vehicle</th>
<th>MTX</th>
</tr>
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<tbody>
<tr>
<td>Duodenum</td>
<td>* - 3</td>
<td>* - nil</td>
</tr>
<tr>
<td></td>
<td>$ - 2 to 3</td>
<td>$ - 1</td>
</tr>
<tr>
<td>Jejunum</td>
<td>* - nil</td>
<td>* - nil</td>
</tr>
<tr>
<td></td>
<td>$ - 1 to 2</td>
<td>$ - nil</td>
</tr>
<tr>
<td>Ileum</td>
<td>* - nil</td>
<td>* - nil</td>
</tr>
<tr>
<td></td>
<td>$ - nil</td>
<td>$ - nil</td>
</tr>
</tbody>
</table>

*apoptotic cells in villi epithelium; $ apoptotic cells in connective tissue of lamina propria.

Parts of small intestine in vehicle treated rats showed normal morphology with sharp villi, long crypts and intact lining epithelium with normal mucus secreting cells, normal vasculature & normal cellularity lamina propria. No edema was seen in any of the components in the wall. This suggests that the administration of the vehicle does not disturb the mucosal integrity in these rats.

MTX treated rats showed necrotic mucosa affecting the villi and crypts, blood vessels and connective tissue of the lamina propria. The villi were bloated and tips were blunted; nevertheless, the villi were free without adhesions; no obvious vascular congestion in the connective tissue and in many places the mucosa was denuded without lining epithelium (Figure 6.3.4). However, signs of proliferation of cells were noticed. The degree of destruction was the same through the length of small intestine. If graded on a six point scale with ‘1’ for normal picture and ‘6’ for highest damage, the necrosis level for the two groups can be given as vehicle treated group: 1, and MTX treated group: 4.
Figure: 6.3.4. Mucosal damage of the small intestine 24 hours after the final dose of MTX.

A. Control rat ileum showing normal morphology with no mucosal damage. *s denote normal villi and normal crypts. There was no vascular damage / congestion or edema in the connective tissue.

B. Higher power magnification of a jejunum segment of small intestine of control rat. Normal morphology of simple columnar lining epithelium (e); mucous secreting goblet cells (*); connective tissue in lamina propria (c); blood vessel (v).

C. MTX produced a disturbing effect of the mucosal wall (chemical induced mucositis). The jejunum section showing blunted villi (arrow head), desquamation of villi epithelial cells (s), increased cellularity (c); edematous blood vessels (double arrow heads); and adhesion of necrotic villi (long arrows).

D. Higher power magnification of duodenum segment of MTX treated rat showing nuded villi without lining epithelium (short arrow), necrotic cells (white star) and rare apoptotic cell (long arrow). b- blood vessels.
6.3.4. Discussion

Chemotherapy commonly produces structural and functional damage to the intestinal mucosa in cancer patients (445) and in patients with rheumatoid arthritis (446). During chemotherapy, the balance between epithelial cell proliferation, differentiation and cell death at the villus tips is disrupted by premature death of the dividing epithelial cells (376). As a result of mucosal barrier injury, a common side effect of MTX chemotherapy is severe mucositis and enterocolitis.

Both constitutive and inducible isoforms of nitric oxide synthase (NOS) are present within the gastrointestinal tract (414, 447-449). NOS can function as a NO – generating or as an $\text{O}_2^-$ generating enzyme, the latter being a result of uncoupling of oxygen reduction and arginine oxidation by NOS (450-453). Low levels of NO produced by the constitutive isoforms of NOS may play a beneficial or homeostatic role in the gastrointestinal tract. On the other hand, sustained release of NO as a result of iNOS up regulation may lead to cellular injury and gut barrier failure. Nitric oxide can be cytotoxic when produced in large amounts and is suggested to play a regulatory role in gut barrier function (412, 421, 454). The increased vulnerability of villus epithelium to injury coupled with an ability to constitutively and rapidly up regulate NO synthesis suggests the intriguing possibility that iNOS plays an important role in acute epithelial defense or repair. Accordingly, in the present study, basal expression of nitrotyrosine (an indirect indicator of iNOS activity) was observed in the apical region of the villi epithelial cells as well in the glandular epithelium, in the connective tissue and in muscle tissue of all the three parts of small intestine (duodenum, jejunum and ileum) of control rats suggesting constitutive production of NO.
In the present study, increased nitrotyrosine level was observed in the villi epithelium of MTX treated rats as compared with control. This increase may contribute to epithelial defense/repair after injury by MTX (or) be responsible for mucosal injury caused by MTX. The pathogenicity of increased NO is suggested to be dependent on the formation of peroxynitrite, a more reactive and toxic metabolite than nitric oxide (140, 455). Co-localization of 3-nitrotyrosine immunostaining and epithelial apoptosis is reported in the intestinal villi under inflammatory conditions suggesting the important role of peroxynitrite induced apoptosis of enterocytes in gut barrier failure (432, 456). Our observation that crypt epithelium did not stain much for basal nitrotyrosine is in agreement with the findings of others (414, 415, 417), who have shown absence of iNOS in the crypt epithelium.

Besides, the nitrate level in the small intestine of MTX treated rats was increased several fold as compared with the control. The increase in tissue nitrate levels and staining for nitrotyrosine was accompanied by neutrophil infiltration of the small intestines, as evidenced by a marked increase in MPO activity, a sensitive indicator of neutrophil infiltration. Our findings suggest that nitric oxide combines with superoxide (derived from activated neutrophils) to form peroxynitrite that nitrates the tyrosine moieties of proteins. The increased peroxynitrite production may be responsible for the loss of absorptive function and barrier function facilitating the entry of bacteria into the epithelium. It is important to recollect that overproduction of nitric oxide and its metabolite peroxynitrite are suggested to play an important role in gut barrier failure and inflammation (412).

It is suggested that peroxynitrite may induce enterocyte apoptosis through several mechanisms including inhibition of mitochondrial function and ATP depletion resulting in cell death (457). Previous studies have shown increased immunohistochemical staining of nitrotyrosine in the
epithelial cells in the inflammatory conditions such as inflammatory bowel disease (IBD) and crohn’s disease (426). In this study, MTX caused increased levels of nitrotyrosine staining in the small intestine.

PARP is an abundant, chromatin bound enzyme constitutively expressed in most cell types (458). It is an energy consuming enzyme thought to be involved in DNA repair (459). PARP activation followed by elevated poly (ADP – ribose) levels is suggested in the release of apoptosis-inducing factors from the mitochondria (460, 461). Cleavage of PARP is a recognized marker of cell death (462). In the present study we have demonstrated constitutive expression of PARP in the epithelium of small intestines of control rats. This suggests that constitutive expression of PARP may have function in the small intestines. It has been demonstrated earlier that PARP-1 is expressed in normal intestinal epithelial cells (463) and that PARP – 1 acts as a survival factor for intestinal stem cells in vivo at low DNA damage levels. In stark contrast, there is a risk of necrotic cell death due to severe NAD⁺ and ATP depletion when PARP – 1 is acutely over–activated (435).

Poly (ADP-ribose) synthetase activity was shown to be present in the nuclei of crypt intestinal epithelial cells undergoing mitotic division. The enzyme activity is known to be almost absent in the differentiating upper crypt cells and villus cells that are not participating in the cell division cycle in normal intestinal epithelium in vivo. The size of poly (ADP-ribose) chains attached to nuclear proteins in cells at the bottom of intestinal crypts does not alter during differentiation and migration of the cells on to the intestinal villi, but a narrower range of sizes of poly (ADP-ribose) chains are synthesized in villus nuclei. Evidence exist for the ability of both crypt and villus cell nuclei to initiate the ADP – ribosylation of nuclear proteins in vitro, but ADP – ribosyl units are
incorporated into these proteins from NAD$^+$ predominantly by elongation in vitro of oligo(ADP–ribose) chains which preexisted on nuclear proteins in vivo (464).

In our study both the villi cells and the crypt cells immunostained uniformly for PARP in the normal rat intestines. The results could reflect the numerically dominant villus nuclei as compared with crypt. It is noteworthy to mention that villus cells account for about 75%, crypt cells for about 25% of total epithelium (465). The cells migrating from the site of proliferation at the base of the crypt to the tip of the villi before they undergo apoptosis and shed could also contribute to PARP staining in the tip of the villi in normal epithelium.

In this study, unlike in control rats, immuno expression of PARP was erratic, focal and not uniform in the small intestines of MTX treated rats. The increased intensity of focal PARP was confined to the tip of the villi. This suggests that in the MTX treated rats, PARP is activated almost exclusively in the villi epithelial cells, resulting in necrosis of villi cells mainly. As villi epithelial cells are involved in absorption of nutrients, their damage and death may be responsible for weight loss (due to malabsorption of nutrients), and diarrhea in the MTX treated rats.

The mechanisms underlying the choice of PARP 1–dependent cell death pathways (i.e., necrosis vs. apoptosis) in response to genotoxic stimuli have not been determined, but may be influenced by the type, strength, and duration of the stimuli, as well as the cell type (221). One feature of apoptosis is its dependency on ATP for the ordered degradation of cellular structures and maintenance of membrane integrity (219). Thus, in cells whose ATP pools have been depleted due to PARP–1 activation, cell death occurs by necrosis.
Evidence from studies utilizing cultured cells has demonstrated that ROS produce strand breaks in DNA that trigger the activation of the nuclear enzyme poly(ADP ribose) polymerase (PARP) (459). Once activated, PARP catalyzes the transfer of ADP ribose moieties from NAD to nuclear proteins, including histones, and onto PARs itself (auto modification), with the concomitant formation of nicotinamide (201). However, there is now good evidence that exposure of cells to oxidant stress (e.g. \( \text{H}_2\text{O}_2 \), peroxynitrite, etc.) results in strand breaks in DNA, leading to an excessive activation of PARP that results in the depletion of its substrate NAD in vitro and a reduction in the rate of glycolysis (466, 467). As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP levels (468-471). Furthermore, nicotinamide formed by PARP activation can be recycled back to NAD via a mechanism that also consumes ATP (469). Thus, activation of PARP leads to a fall in ATP via two different mechanisms, leading to cellular dysfunction and ultimately cell death. Overall, this process has been termed the “PARP Suicide Hypothesis” (471). There is recent evidence that the activation of PARP plays an important role in shock and inflammation (215, 472).

Accordingly, in the current study PARP expression was increased and villus cell necrosis was observed in the small intestines of MTX treated rats as compared with controls. Besides, increased oxidative stress and nitrosative stress were observed in the small intestines of MTX treated rats as compared with control. These results suggest that increased oxidant stress induced by MTX may activate the suicide cycle of PARP, deplete ATP resulting in villi epithelial cell death by necrosis.

Methotrexate treatment has been shown to result in cleavage of PARP in Hec59 cells (473). The activation of PARP observed in response to MTX is thought to be because of oxidative damage
to DNA. PARP inhibition or PARP inactivation has been shown to reduce the formation of nitrotyrosine – an indicator of the formation of peroxynitrite – in the inflamed tissues (141, 474-476). Recent study has shown that inhibition of PARP activation decreased the mucosal injury and also preserved the mucosal barrier function in small intestine of rat (477).

In the present study, apoptotic cells were observed in the villus epithelium of control (normal rats) as well as in MTX treated rats. It is not surprising to find apoptotic cells in normal rat villi epithelium as apoptosis is normal phenomenon by which the epithelial cells are shed. Intestinal epithelial cells (IEC) derived from stem cells at the base of the crypts, migrate along the crypt villus axis, and reach the lumen after a life span of only 3–5 days (478, 479). At the luminal surface, they die of apoptosis, lose anchorage, detach, and are shed (480, 481). There was no difference in the number of apoptotic cells between the villus epithelium of MTX treated rats and normal rats. This suggests that MTX induced destruction of small intestinal mucosa is not by apoptosis. However, necrosis was clearly evident in all three parts of small intestine. In other words, MTX induce destruction of small bowel mucosa not by apoptosis but by necrosis.

It was observed recently that exposure of endothelia and epithelial cells to H$_2$O$_2$ result in ADP–ribosylation, NAD$^+$ depletion, inhibition of mitochondrial respiration, and increased paracellular permeability. The administration of PARP inhibitor, 3–aminobenzamide has been shown to provide a significant, partial protection against the energetic and functional changes in a rat model of endotoxic shock (472, 482).

From our findings thus far, we propose that MTX causes increased oxidative stress and nitrosative stress in the small intestines of rats, thereby triggering PARP activation. PARP activation in turn results in NAD$^+$ and ATP depletion in the villus epithelial cells resulting in
necrosis. As villi epithelial cells are involved in absorption of nutrients, their damage and death can result in malabsorption of nutrients, weight loss, and diarrhea in the MTX treated rats.

In conclusion, the results of the present study suggest that PARP activation and nitrotyrosine formation are important events in MTX – induced small intestinal damage, and that villus epithelial cell necrosis may be due to PARP activation. This is the first report to show that nitrosative stress and PARP activation play a role in MTX – induced small intestinal damage, to the best of our knowledge.
STUDY IV

Effect of methotrexate on mitochondrial structure and function of enterocytes
Abstract

MTX is known to cause small intestine damage, particularly to the enterocyte mitochondria. Previous studies have shown that MTX treatment decreases the activity of succinate dehydrogenase and as well as the mitochondrial content. This study was aimed to know the effect of MTX on the structure and function of mitochondria in the enterocytes at different stages of maturation and differentiation in the small intestine of rat. MTX was administered at 7mg/kg body weight intraperitoneally for 3 consecutive days. 12 hours and 24 hours after the final dose of MTX, the animals were sacrificed and the entire length of small intestine was removed. Different enterocytes population (villus, middle and crypt cells) was isolated and homogenized. The homogenates were used for isolation of mitochondria. The isolated mitochondria were used in the experiments to assess the mitochondrial damage by measuring various parameters of mitochondrial function such as RCR, MTT reduction, mitochondrial swelling and the activities of the mitochondrial electron chain complexes I, II, III, IV and V.

For electron microscopy, rats were administered with a single dose of 25 mg/ kg body weight intraperitoneally and were sacrificed after 2hrs, 6hrs, 12hrs, 24hrs and 48hrs.

The results showed that MTX administration decreased RCR and MTT reduction and caused mitochondrial swelling. The activities of complex II and complex IV were significantly decreased and the activity of complex V was increased significantly as compared with the controls. The cristae of mitochondria were damaged as early as 6hrs in jejunum and ileum.

These studies showed that treatment with MTX had lead to mitochondrial damage and dysfunction by altering the activity of mitochondrial electron chain complexes in the villus, middle and crypt cells of the small intestine.
6.4.1. Introduction

MTX is known to alter the morphology of mitochondria as suggested in the previous reports (19, 25). The functional alteration of mitochondria after treatment with MTX has not been reported except for the decreased activity of succinate dehydrogenase (26). Mitochondria are the major source of free radicals and the excessive production of free radicals may lead to loss of mitochondrial function and cell death. Mitochondrion embodies its functions through: [1] production of ATP; [2] production of ROS, which regulates nuclear expression; [3] linking cytoplasm and nucleus; [4] sensitive response to stimulator or signals outside cytoplasm; [5] triggering of cell death (483, 484). Evidences show the central role of mitochondria in the development of apoptosis (485-487).

Mitochondria produce ATP constantly and are therefore permanently active. The proton gradient that drives the ATP synthase (which generates the majority of ATP normally used by cells) is generated by the transfer of electrons along the respiratory (electron transport) chain. The components of the chain are therefore constantly switching from their oxidized to reduced forms and back again as the electrons travel along it at a rate that has been calculated to be approximately 60 electrons per second (488). The parameters that were used to assess mitochondrial functions are respiratory control ratio (RCR - ratio of oxygen consumption in state III/state IV respiration), MTT reduction assay, mitochondrial permeability transition (mitochondrial swelling), calcium flux and the activities of the mitochondrial electron chain complexes I – V.

Respiratory control ratio (RCR) and activity of complex IV are considered as indicators of mitochondrial respiratory function. RCR indicates the tightness of the coupling between respiration and phosphorylation. RCR is directly related to the viability of mitochondria. RCR is
found to be decreased in conditions of mitochondrial damage. Cytochrome c oxidase (complex IV of the mitochondrial electron transport chain) is the primary site of cellular oxygen consumption and, as such, is central to oxidative phosphorylation and the generation of ATP (489). Cytochrome c oxidase (CcO) contains two heme (a and a$_3$) and two copper (Cu$_A$ and Cu$_B$) centers, of which the heme iron of cytochrome a$_3$ together with Cu$_B$ (in their reduced form) constitute the binding site for oxygen. It is located on the inner membrane of the mitochondrion and catalyzes the oxidation of cytochrome c and the reduction of oxygen to water. This process is linked to the pumping of protons into the mitochondrial inter membrane space. In this last step, groups of four electrons participate in a cycle in which the oxygen-binding site of CcO is alternately reduced and oxidized in the process of generating water from oxygen and protons to drive the ATPase. When there is sufficient oxygen, the enzyme predominates in its oxidized state while, as the oxygen decreases and becomes limiting, the enzyme is more abundant in its reduced state.

MTT reduction assay is a measure of mitochondrial activity as well as cell viability. In the MTT assay, MTT is cleaved to formazan by the “succinate – tetrazolium reductase” system of the mitochondrial respiratory chain and is active only in the viable cells (490). Cellular damage will inevitably result in loss of the ability of the cell to maintain and provide energy for metabolic function and growth and so the extent of reduction of MTT will be decreased.

A well known index of mitochondrial dysfunction is represented by changes in the permeability of the mitochondrial membranes, which may affect occurrence of the mitochondrial permeability transition (MPT). MPT is a physiopathological event that results in increased permeability of the inner mitochondrial membrane to solutes with a molecular mass of <1.5 kDa resulting in mitochondrial swelling. Mitochondrial swelling is considered as an indicator of altered MPT.
MPT can also disturb the mitochondrial functions in several ways by affecting the cellular bioenergetics, cell survival, and proliferation (122, 491-493). It is now largely accepted that MPT is due to opening of the MPT pore (MPTP) with subsequent release of cations, mitochondrial proteins with subsequent loss of mitochondrial transmembrane potential followed by mitochondrial swelling, and apoptosis (483, 491). The MPTP comprises of several proteins such as cyclophilin D (CypD) and the ATP/ADP translocator adenine nucleotide translocater (ANT) (494), and its opening is finely regulated by a plethora of factors, including changes in Ca\(^{2+}\), pH, reactive oxygen species (ROS), ADP/ATP levels and the expression levels of Bcl-2, an antiapoptotic protein (494, 495). Mitochondrial permeability transition (MPT) is now generally accepted as irreversible mitochondrial damage leading to mitochondrial calcium perturbation, mitochondrial swelling, and ROS production and release (496-498). The attack of ROS to membrane protein thiols produces cross linking reaction that may open membrane pores (499). The opening of MTP pore due to ROS and cytokines was first described in necrotic cell death (500, 501). It is now considered that the mitochondrial permeability transition (MPT) plays a key role in the pathogenesis of apoptosis and necrosis. If MPT initiates rapidly with depletion of ATP, cells undergo necrosis, and if MPT occurs without depletion of ATP, cells evolve into apoptosis (493, 500, 502).

The mitochondrial respiratory chain has been recognized as one of the major site of ROS production, and therefore a major source of oxidative stress (503). Early evidence, mainly from studies using the isolated organelle, suggested that a small percentage (2 – 4 %) of the oxygen used by the electron transport chain is not completely reduced to water but is instead converted to superoxide anion (O\(_2^-\)). This occurs as a result of the escape of electrons at complexes I and III of the respiratory chain (504-508). More recent experiments support the view that the release of
ROS from mitochondria is dependent on complex III (509-511). The superoxide produced in the mitochondria is immediately dismutated by the mitochondrial superoxide dismutase to produce hydrogen peroxide. Hydrogen peroxide interacts with metals such as iron to form highly reactive hydroxyl radicals (512). The ROS are normally metabolized by the cell’s antioxidant system, and a certain level of ROS is even needed in normal cell functions i.e., signal transduction pathways (513). Excessive ROS production or inadequate antioxidant protection can occur and can cause damage to cell structures like lipids, proteins, and DNA (512) or induce cell death (514). The mitochondrial ‘vicious cycle’ theory implies that ROS production during respiration is associated with increased oxidative damages leading to mitochondrial dysfunction that further increases ROS production (515). NO is known to react with O$_2^-$ to form more potent peroxynitrite that causes damage to the mitochondrial complexes (195). Recent study had suggested the production of NO by mitochondria by a constitutive calcium sensitive mitochondrial nitric oxide synthase (135). NO is known to inhibit cytochrome oxidase and mitochondrial respiration (192). Peroxynitrite has been shown to inhibit complex I and complex II dependent mitochondrial respiration, but had not affected cytochrome c oxidase dependent oxygen consumption (164, 516). Thus, it is suggested that MTX induced increased NO production might affect the mitochondrial respiration in the small intestine.

Very little is known about the effect of MTX on the mitochondria of small intestine. An earlier study has shown a decrease in the number, size and stainability of the mitochondria (26). In contrast, Bessler et al had reported a marked increase in size of mitochondria in the small intestine following methotrexate treatment (25). The effect of high dose of MTX on the mitochondria of small intestine in a short period of time is not known. Hence, the experiments
were designed to study the effect of MTX on the morphological and functional alterations of mitochondria in the villus, middle and crypt cells of small intestine in rat model.

6.4.2. Summary of experiment

Adult male Wistar rats were given 7 mg /kg body weight MTX intraperitoneally for 3 consecutive days. The animals were sacrificed 12 hours and 24 hours after the final dose of MTX. The entire length of small intestine was removed, washed with ice cold saline, and the intestinal epithelial cells were isolated as intact cells from different levels of the villus, middle and crypt areas representing sequential stages of cellular differentiation (322). The 9 fractions obtained were pooled into 3 as follows: fractions 1 – 3 were pooled and consisted predominantly of villus tip cells, 4 – 6 formed the middle cell fraction and 7 – 9 were pooled to form the crypt cells. The different enterocyte population was homogenized and used for the isolation of mitochondria (323). The purity of the isolated mitochondria was assessed by enrichment of the marker enzyme succinate dehydrogenase. The isolated viable mitochondria were used to assess the mitochondrial damage by measuring various parameters of mitochondrial function such as RCR, MTT reduction, mitochondrial swelling and the activities of the mitochondrial complexes I, II, III, IV and V.

For electron microscopy, a single dose of 25 mg/ kg body weight was given intraperitoneally and the rats were euthanized after 2h, 6h, 12h, 24h and 48h. The small intestines were removed, flushed with cold PBS and divided in to duodenum, jejunum and ileum. The sections were fixed in 2.5% glutaraldehyde, post fixed in osmium tetroxide and embedded in araldite (epoxy resin). One micron thick sections were cut and stained with toluidine blue. The selected areas were examined under a Philips EM201C electron microscope (Eindhoven, The Netherlands).
6.4.3. Results

i. Effect of MTX on the structure and function of mitochondria in the enterocytes of small intestine of rat

Rats treated with methotrexate showed altered mitochondrial function. The respiratory control ratio (RCR) was significantly decreased at 12 hours in villus cells’ mitochondria (p value < 0.004) and at 24 hours in middle cells’ mitochondria (p value < 0.002) and crypt cells’ mitochondria (p value < 0.005) as compared to controls (Figure: 6.4.1). The MTT reduction by mitochondria was significantly decreased at 12 hours in villus cells’ mitochondria (p value < 0.05) and at 24 hours in middle cells’ mitochondria (p value < 0.04) and crypt cells’ mitochondria (p value < 0.01) as compared to controls (Figure: 6.4.2). The middle cells’ mitochondria showed significantly increased swelling as evidenced by decreased absorption at 540 nm at 24 hours as compared to controls (Figure: 6.4.3).

There was no significant difference in the activity of complex I after treatment with MTX as compared to control (Figure: 6.4.4). The activity of complex II was significantly decreased in the villus, middle and crypt cells after treatment with MTX as compared to control (Figure: 6.4.5). There was no significant difference in the activity of complex III after treatment with MTX as compared to control (Figure: 6.4.6). The activity of complex IV was significantly decreased in the villus, middle and crypt cells after treatment with MTX as compared to control (Figure: 6.4.7). The activity of complex V was increased slightly in villus and middle cells as compared to controls (Figure: 6.4.8).
Respiratory control ratio (RCR) of mitochondria isolated from different enterocytes fractions from control and MTX – dosed rats at 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group), * P < 0.01 as compared with control.
MTT reduction of mitochondria isolated from different enterocytes fractions from control and MTX – dosed rats at 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group), * P < 0.05 as compared with control.
Change in absorbance of mitochondria isolated from different enterocytes fractions from control and MTX-dosed rats at 12 hours and 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group), * P < 0.05 as compared with control.
Complex I activity of mitochondria isolated from different enterocytes fractions from control and MTX-dosed rats 24 hours after the final dose of MTX. Data represent mean ± SD, n = 6 (control group); n = 8 (MTX group).
Complex II activity of mitochondria isolated from different enterocytes fractions from control and MTX-dosed rats 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group). * P < 0.01 as compared with control. ** P < 0.001 as compared to control.
Complex III activity of mitochondria isolated from different enterocytes fractions from control and MTX-dosed rats 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group).
Complex IV activity of mitochondria isolated from different enterocytes fractions from control and MTX-dosed rats 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group), * P < 0.01 as compared with control.
Complex V activity of mitochondria isolated from different enterocytes fractions from control and MTX-dosed rats 24 hours after the final dose of MTX. Data represent mean ± SD, n= 6 (control group); n = 8 (MTX group), * P < 0.05 as compared with control.
ii. **Effect of MTX on the ultra structural changes in the small intestine of rat**

In the duodenum, the cristae of mitochondria were damaged at 24 hours and 48 hours after MTX treatment as compared to control (Figure: 6.4.7 A–C). At 48 hours, the microvilli were found to be damaged (Figure: 6.4.7 D & E). The endoplasmic reticulum and golgi apparatus were found to be dilated as a degenerative vacuolar appearance (Figure: 6.4.7 F).

In the jejunum, disruption of cristae of the mitochondria was seen at 6 hours, 12 hours, 24 hours and 48 hours after MTX treatment as compared to control (Figure: 6.4.8 A–E). Dilatation of endoplasmic reticulum and golgi apparatus was seen as a degenerative vacuolar appearance at 24 hours after MTX treatment (Figure: 6.4.8 F).

In the ileum, cristae of the mitochondria were disrupted at 6 hours, 12 hours, 24 hours and 48 hours after MTX treatment as compared to control (Figure: 6.4.9 A–E). The microvilli were found to be damaged at 48 hours (Figure: 6.4.9 F & G). Dilatation of endoplasmic reticulum and golgi apparatus was seen as a degenerative vacuolar appearance at 24 hours after MTX treatment (Figure: 6.4.9 H).
Electron microscopic pictures of duodenum of rat with the arrows showing

A) Normal mitochondria seen in control, 29120 X.
B) Cristae of mitochondria damaged at 24 hrs, 13867 X.
C) Cristae of mitochondria damaged at 48 hrs, 41700 X.
D) Normal microvilli of control. 1410 X.
E) Damaged microvilli after 48 hrs of MTX treatment, 4172 X.
F) Focal dilatation of endoplasmic reticulum and golgi apparatus giving rise to a degenerative vacuolar appearance 48 hrs after MTX treatment, 5960 X.
Electron microscopic pictures of jejunum of rat with the arrows showing

A) Normal mitochondria seen in control, 27300 X.
B) Cristae of mitochondria damaged at 6 hrs after MTX treatment, 27300 X.
C) Cristae of mitochondria damaged at 12 hrs after MTX treatment, 27800 X.
D) Cristae of mitochondria damaged at 24 hrs after MTX treatment, 27300 X.
E) Cristae of mitochondria damaged at 48 hrs after MTX treatment, 31850 X.
F) Focal dilatation of endoplasmic reticulum and golgi apparatus giving rise to a degenerative vacuolar appearance 24 hrs after MTX treatment, 30940 X.
Figure: 6.4.9

Electron microscopic pictures of ileum of rat with the arrows showing

A) Normal mitochondria seen in control, 9600 X.
B) Cristae of mitochondria damaged at 6 hrs after MTX treatment, 36400 X.
C) Cristae of mitochondria damaged at 12 hrs after MTX treatment, 27300 X.
D) Cristae of mitochondria damaged at 24 hrs after MTX treatment, 47657 X
E) Cristae of mitochondria damaged at 48 hrs after MTX treatment, 47260 X.
F) Normal microvilli seen in control, 5227 X.
G) Damaged microvilli seen 48 hrs after MTX treatment, 2010 X
H) Focal dilatation of endoplasmic reticulum and golgi apparatus giving rise to a degenerative vacuolar appearance 24 hrs after MTX treatment, 7467 X.
6.4.4. Discussion

Electron microscopic studies reveal that MTX- damages the mitochondria of enterocytes. MTX is known to affect oxidative phosphorylation in isolated liver mitochondria and little is known about the effects of MTX on the mitochondria of small intestine. MTX is known to induce ROS-dependent mitochondrial apoptosis in different cell lines (116, 517-520). Though there are some previous reports describing the morphological changes in mitochondria by MTX in the small intestine, the effect of MTX on the function of mitochondria in small intestine is not clear. Hence, the experiment was designed to study the effect of MTX on the structure and function of mitochondria in the enterocytes of small intestine at different stages of maturation and differentiation.

First, the effect of MTX on enterocyte cell viability was studied. In the MTX treated rats, the cell viability of enterocytes was significantly decreased as compared with control. The decrease in cell viability, seen in this study in response to MTX may be attributed to increased oxidative stress as reported in the earlier part of our work and may be detrimental to the functioning of cells.

Then, the mitochondrial respiratory function was evaluated by assessing the activity of complex IV, a key mitochondrial respiration enzyme, RCR and mitochondrial swelling. Evidence of impairment of mitochondrial function was evident in the present study, as seen by the lowered RCR, fall in absorbance at 540 nm and decreased activity of complex IV. Decrease in RCR is indicative of a disturbance of mitochondrial respiratory function. Decreased complex IV activity may be responsible for decreased mitochondrial respiration. Complex IV inhibition is predicted to increase ROS production, initially superoxide anion, from electrons lost from the ETC (521). Complex II is considered as a source of ROS (522, 523). Inhibition of complex II is
accompanied by a biphasic increase of reactive oxygen species (ROS) and concurrent glutathione oxidation, in addition to a slight decrease in the cellular ATP level (524). In the current study, MTX had significantly inhibited the activity of complex II in agreement with the previous study which also showed decreased activity of complex II in the jejunal mucosa of mice in response to MTX (525). Thus, it is concluded that MTX inhibits complex II thereby increases the ROS production (or) it may be that under oxidative stress conditions caused by MTX, the activity of complex II was reduced in order to avoid further production of ROS. The activity of complex V was slightly increased in the enterocyte mitochondria of MTX treated rats, the reason of which is unclear. In the MTX treated rat enterocytes, despite the increased activity of complex V that synthesizes ATP, necrosis was observed. Although there is no clear explanation for this, we suggest that the ATP synthesized by the enterocytes is utilized for the activation of PARP thereby decreasing the availability of ATP for other vital processes in the enterocyte, resulting in necrosis.

An earlier study on the effect of MTX on liver mitochondria showed that MTX has no effect on the activity of ETC complexes (114). These authors have suggested that the inhibition of mitochondrial respiration by MTX is not linked to respiratory chain and that it inhibits NAD$^+$–dependent dehydrogenases of citric acid cycle (114, 115). Thus MTX seems to affect mitochondrial function in different ways in different cells.

The mitochondria obtained from MTX treated rat intestines showed a fall in absorbance at 540 nm. The fall in absorbance at 540 nm is a consequence of swelling of the mitochondria that is in turn due to increased permeability of the inner mitochondrial membrane to small molecular weight solutes. Mitochondrial membrane permeabilization (MMP) is a central event in apoptosis which can be mediated through mitochondrial permeability transition pore (MPTP) opening
The pore is thought to exist as a multiprotein complex, currently of unknown composition (528), that spans both the outer and inner mitochondrial membranes. The opening of the mitochondrial permeability transition pore (MPTP) (529) or damage to the respiratory chain causes failure of the very processes that develop and sustain the potential resulting in the gradual loss of potential due to proton leak. With the involvement of NO established in the damage of mitochondrial membrane and in the opening of MPTP (530), either mechanism seems plausible. In isolated mitochondria, NO is known to compete with O$_2$ at cytochrome c oxidase (530, 531) and so may suppress respiration, and also cause irreversible nitrosylation at complex I (532). Conditions including high calcium (Ca$^{2+}$) and oxidative stress are known to trigger MPTP opening, causing a sudden influx of small molecules including H$_2$O, resulting in collapse of the membrane potential, leading to mitochondrial swelling and rupture of the outer membrane, followed by the release of pro-apoptotic proteins (i.e., cytochrome c, apoptosis inducing factor and endonuclease G) residing in the inter membrane space (533). The released cytochrome c complexes with apoptosis protein activator factor-1 (Apaf-1), dATP and procaspase-9 and forms an apoptosome, which then activates caspase – 9. This latter, in turn, cleaves and activates caspase-3, -6, and -7, main executioners of cell death (533).

The results of this study indicate that MTX nonspecifically alters the mitochondrial function in all the different enterocyte population of small intestine. The increased susceptibility of enterocytes in response to MTX may possibly be due to mitochondrial dysfunction mediated through excessive production of nitric oxide, oxidative stress and mitochondrial membrane damage (534).

Mitochondrial dysfunction, in addition to causing a fall in cellular ATP levels, can result in increased generation of oxygen free radicals. ROS are produced mainly at respiratory complexes
I and III in the form of the superoxide anion (O$_2^-$) that rapidly converts into hydrogen peroxide (506). Mitochondria are not the only sources of ROS. The other sources of ROS in the cell are the radical-generating enzymes xanthine oxidase (535) and NADPH oxidase (536), and activated neutrophils (537). Phospholipase A2 - activated arachidonic acid metabolism can also be a source of ROS (538). In our previous study, and studies reported by others, it has been shown that neutrophil infiltration occurs in the small intestines of rats that are treated with MTX. In the small intestines of MTX treated rats there is overproduction of superoxide anion by the activated neutrophils that accumulate in the gut mucosa (318, 394, 539).

Oxidative stress is known to impair mitochondrial respiration by increase in the production of ROS (mainly O$_2^-$), and decrease in mitochondrial SOD (540, 541). We have demonstrated significant decrease in the activity of SOD in the MTX treated rat enterocyte mitochondria (542). The combination of oxidative and nitrosative stress, leading to peroxynitrite formation, is known to result in the irreversible inhibition of mitochondrial respiration through the iron-sulfur centers of the respiratory chain (543). We have demonstrated MTX – induced increased production of reactive oxygen species and reactive nitrogen species (539, 542). Thus, an excess of both ROS and RNS may damage mitochondria and lead to energy depletion and cell death. The inactivation or inhibition of the ETC complexes might also be a cellular protective mechanism against the lethal effects of oxygen free radicals as suggested earlier. Free radical induced damage can render mitochondrial DNA nonfunctional and the mitochondria functionally defective resulting in respiratory deficiency and the cells lacking mitochondrial genome were found to be resistant to cell death by ROS and oxidative stress (544).
Mitochondria can also be a target of attack by free radicals. Mitochondrial membrane proteins and lipids have been shown to undergo damage in response to exposure to free radicals (534). Such damage to these membranes has been reported to lead to apoptosis (545).

The gastrointestinal tract is the primary site for nutrient absorption and transport. Cellular ATP generated in mitochondria via oxidative phosphorylation fuels nutrient absorption and transport in the small intestine. A considerable amount of energy is used by the gut for the maintenance of this tissue because intestinal epithelium is continuously renewed. New cells are produced in the crypts, migrate to villus tip, and are sloughed off in 3-5 days. Bride and Kelly (1990) estimated that 11-18 % of the whole energy expenditure in ruminants is used by the gut, and the majority of this energy is spent for Na⁺-K⁺ ATPase activity (6 – 12 %), and protein synthesis (4.0 to 4.6 %) (546). For this reason, the absorptive capacity of the small intestine is directly influenced by the availability of ATP to fuel Na⁺K⁺ ATPase and to renew epithelial cells.

The contribution of the gastrointestinal tract to the whole body oxygen consumption has been reported to be approximately 20% in ruminants (546). Inefficiencies in mitochondrial function caused by methotrexate observed in our study may limit nutrient absorption resulting in weight loss. In agreement with other studies, our study showed significant loss of body weight (6%) of rats in response to MTX when compared with the control (24, 356, 366, 405). The weights of the small intestine were significantly reduced (by 38%) in the MTX treated rats as compared with controls.

Several studies have shown that absorptive function of the intestine injured by MTX is reduced (23, 317, 357). Erdman et al (547) have shown a significant decrease in 3–O–methyl–D glucose absorption and maltase – specific activity in the MTX treated rat. Yamamoto et al (303) studied
the permeability of D-glucose, D-xylose and L-leucine through the small intestine of rats treated with MTX using everted segments of small intestine. They observed that MTX treatment decreased the permeability of these nutrients and also lowered the bioavailability of D-glucose. The standard D-xylose absorption test carried out on rheumatoid arthritis patients treated for at least 12 months with methotrexate was significantly decreased indicating intestinal malabsorption (446).

With a single dose of 25mg/ kg body weight, mitochondrial changes were seen as early as 6 hours and the damage to the mitochondria was more at 48 hours after methotrexate administration. This is the first study to report mitochondrial damage by MTX at an early time point of 6 hours after MTX treatment. The focal dilatation of golgi and ER were also observed in the enterocytes of MTX treated rats in agreement with the earlier study done on jejunal biopsy of children with acute lymphoblastic leukemia on methotrexate treatment (108).

In conclusion it is stated that methotrexate treatment induces impairment in mitochondrial function of the enterocytes as evidenced by decreased activity of complexes II and IV, decreased RCR, as well as swelling of mitochondria. The impairment in mitochondrial function may be due to a direct effect of MTX on the mitochondria, or an indirect action of the drug causing free radical production that affects mitochondrial function or a combination of these factors. Mitochondrial dysfunction can result in increased generation of oxygen free radicals, oxidative stress and depletion of antioxidants. The increased generation of ROS by the mitochondria can in turn damage the mitochondria and stimulate the mitochondrial pathway of necrosis. It is suggested that mitochondrial dysfunction and the resultant depletion of ATP caused by MTX may limit nutrient absorption in the small intestine, resulting in diarrhea and weight loss.
STUDY V

Effect of pretreatment with melatonin on methotrexate induced oxidative stress, nitrosative stress, and small intestinal damage.
Abstract

The antifolate drug methotrexate induces small intestine damage resulting in malabsorption and diarrhea. The purpose of this study was to investigate whether exogenous melatonin could protect the small intestine from MTX – induced damage in rats. The rats were pretreated with melatonin (20 & 40 mg/kg body weight) one hour before the MTX (7mg/kg body weight) administration for three consecutive days. Twenty four hours after the final dose of MTX, the rats were sacrificed and the sections of small intestine were taken for light microscopy and immunohistochemistry. Homogenate of other intestine segments was used for assay of biochemical parameters by which the extent of oxidative stress, nitrosative stress and neutrophil infiltration were evaluated. MTX significantly increased the tissue levels of MDA, protein carbonyl and nitrate. The activities of myeloperoxidase and glutathione peroxidase were significantly increased and that of GST, glutathione reductase, catalase and SOD were significantly decreased as compared to control. Pretreatment with melatonin reversed all these changes except for myeloperoxidase activity. The immunohistochemical staining for nitrotyrosine and the immuno expression of PARP were found to be increased after methotrexate administration and these changes were found to be reduced upon pretreatment with melatonin. In conclusion, the present study demonstrates that melatonin is capable of reversing MTX – induced oxidative stress, nitrosative stress, and small intestinal damage, indicating that it may be beneficial in ameliorating the symptoms of MTX induced enteritis in humans.
6.5.1. Introduction

Methotrexate (MTX) is used widely as a chemotherapeutic agent in the treatment of malignancies and various inflammatory diseases, such as psoriasis, rheumatoid arthritis and inflammatory bowel disease (9). However, the usage of this drug is often limited by severe side effects and toxic squeal. MTX non-selectively acts on cancer cells as well as the normal tissues that have a high rate of proliferation, including the haematopoietic cells of the bone marrow and the actively dividing cells of the small intestine. Thus, one of the major toxic effects of MTX is intestinal injury and enterocolitis (2, 111). The small intestinal damage induced by MTX treatment results in malabsorption and diarrhea disturbing the cancer chemotherapy of patients (18). Reactive oxygen species are implicated in the pathogenesis of MTX-induced small intestinal damage (107). In our study, we have shown that MTX administration results in increased malondialdehyde (MDA) levels and myeloperoxidase (MPO) activity and altered activities of antioxidant enzymes in the small intestine, thus indicating neutrophil infiltration and oxidative stress in the pathogenesis of MTX-induced small intestinal damage. The protein modification as shown by increases nitrotyrosine staining and the nuclear damage as evidenced by increased PARP staining were also reported in our study. Melatonin has been shown to be a very strong antioxidant and free radical scavenger (548). Melatonin, because of its small size and high lipophilicity, crosses biological membranes easily; this property is suggested to be of possible importance to the hormone inside the cell through the activation of nuclear receptors (549). The gastrointestinal tract of numerous animal species contains melatonin which is synthesized essentially by intestinal enterochromaffin cells (550).

The present study was undertaken to find out whether melatonin, as a potent free radical scavenger, could ameliorate MTX-induced oxidative stress, nitrosative stress, and small intestinal damage.
6.5.2. Summary of experiment

Adult male Wistar rats (200-250g) were divided into six groups.

Group I: The rats in this group (n = 6) received the vehicle alone intraperitoneally for 3 days.

Group II: The rats in this group (n = 6) received 20mg/kg of melatonin alone intraperitoneally for 3 days.

Group III: The rats in this group (n = 6) received 40mg/kg of melatonin alone intraperitoneally for 3 days.

Group IV: The rats in this group (n = 8) received three consecutive daily intraperitoneal injections of methotrexate at the dose 7 mg/kg body weight.

Group V: The rats in this group (n = 8) received 20mg/kg of melatonin one hour prior to MTX administration intraperitoneally for 3 days.

Group VI: The rats in this group (n = 8) received 40mg/kg of melatonin one hour prior to MTX administration intraperitoneally for 3 days.

The rats were sacrificed 24 hours after the final dose of methotrexate. The rats were anesthetized with halothane and the entire length of small intestine was removed and flushed with cold PBS and divided into duodenum, jejunum and ileum. The portion of tissue were fixed in 10% buffered formalin then embedded in paraffin, sectioned and stained with haematoxylin and eosin for examination by light microscopy and immunohistochemistry. Rest of the mucosa was scraped off from the small intestine using glass slide, weighed and homogenized in appropriate buffers for the biochemical assays.
6.5.3. Results

i) Effect of pretreatment of melatonin on MTX induced light microscopic changes in the small intestine

Light microscopic examination of parts of small intestine in vehicle treated rats showed normal morphology with sharp villi, long crypts, intact lining epithelium with normal mucus secreting cells, normal vasculature and normal cellularity lamina propria (Figure: 6.5.1 A – C). MTX caused damage to the architecture of the small intestine (Figure: 6.5.2 A – C). The villi were distorted and blunted in the duodenum (Figure: 6.5.2 A), atrophied and focally absent in the jejunum (Figure: 6.5.2 B) and aborted, flattened, and fused in the ileum (Figure: 6.5.2 C). The crypt abscesses were found in all the three segments of the small intestine suggesting an inflammatory response. The mucosal thickness was reduced accompanied by decreased villus/crypt ratio. There was acute transmural inflammatory infiltrate in the mucosa, submucosa and the muscularis layers.

The administration of 40mg melatonin alone did cause slight alteration in mucosal morphology especially in jejunum. The tips of the villi were broken and mildly blunted. There was mild increase in cellularity of connective tissue. The cell boundary of the epithelium was not clear in some areas and the crypts were normal generally.

Pretreatment with 40mg/kg melatonin before the administration of MTX showed that the damage to the mucosa and the severity of inflammatory response was less as compared to the MTX treated rats. There was less damage to the villi and less crypt abscess were found in the small intestines of melatonin pretreated rats. The villi/crypt ratio was more and the inflammatory infiltrate of the mucosa and muscularis propria was less as compared to the MTX treated rat. Jejunum showed maximum recovery and the ileum showed least recovery with 40mg/kg melatonin pretreatment (Figure: 6.5.2 D – F). In conclusion, pretreatment with 40 mg/kg melatonin had protected the small intestine from the deleterious effects of MTX.
Histology of the duodenum, jejunum and ileum of control (A – C) and MTX (24 hours) treated rats (D – F), magnification 40X.
Figure 6.5.2

Effect of MTX and melatonin (40mg/kg) pretreatment on the small intestine of rat. 40X

- A, B and C represent duodenum, jejunum and ileum of MTX treated rats respectively. The villi were shortened in the duodenum (A), distorted in the jejunum (B) and aborted, flattened, blunted and fused in the ileum (C).
- D, E and F represent the duodenum, jejunum and ileum of Melatonin (40mg/kg) pretreated rats respectively.

Black arrow indicates the villi and the white arrow indicates the crypt abscess.
ii) Effect of melatonin pretreatment on MTX induced changes in parameters of oxidative stress

Pretreatment with 20mg/kg melatonin had significantly abolished the MTX – induced elevated levels of MDA and protein carbonyl content (Figure: 6.5.3 & 6.5.4). Melatonin at a concentration of 20mg/kg had significantly abolished the elevated glutathione peroxidase activity and decreased activity of glutathione reductase (Figure: 6.5.5 & 6.5.7) in response to MTX. The decrease in glutathione S transferase activity was not abolished by 20mg/kg of melatonin pretreatment (Figure: 6.5.6). Instead the glutathione s transferase level was further decreased on pretreatment with melatonin. Pretreatment with 20mg/kg of melatonin had no effect on the increased myeloperoxidase activity (Figure: 6.5.8) and on the decreased activities of catalase (Figure: 6.5.9) and superoxide dismutase (Figure: 6.5.10) in response to MTX.

Pretreatment with 40mg/kg melatonin had also significantly abolished the increased levels of MDA and protein carbonyl (Figure: 6.5.3 & 6.5.4) in response to MTX. Pretreatment with 40mg/kg melatonin had significantly abolished the decreased activity of glutathione reductase (Figure: 6.5.7), but had no effect on the elevated activity of glutathione peroxidase and the decreased activity of glutathione s transferase (Figure: 6.5.5 & 6.5.6). Melatonin at 40mg/kg had significantly restored the activity of superoxide dismutase (Figure: 6.5.10), but had no effect on the increased activity of myeloperoxidase (Figure: 6.5.8) and decreased activity of catalase (Figure: 6.5.9) in response to MTX.
Figure: 6.5.3

Malondialdehyde levels in the small intestines of control rats and experimental rats with melatonin (20mg & 40mg) treatment, MTX treatment and melatonin pretreatment groups. Data represent mean ± SD, n= 6 (control & melatonin groups); n=8 (MTX & melatonin + MTX groups), * P < 0.01 as compared with control group; # p < 0.01 as compared with MTX group.

Figure: 6.5.4

Protein carbonyl content in the small intestines of control rats and experimental rats with melatonin (20mg & 40mg) treatment, MTX treatment and melatonin pretreatment groups. Data represent mean ± SD, n= 6 (control & melatonin groups); n=8 (MTX & melatonin + MTX groups), * P < 0.01 as compared with control group; # p < 0.001 as compared with MTX group.
Glutathione peroxidase activity in the small intestines of control rats and experimental rats with melatonin (20mg & 40mg) treatment, MTX treatment and melatonin pretreatment groups. Data represent mean ± SD, n= 6 (control & melatonin groups); n=8 (MTX & melatonin + MTX groups), * P < 0.05 as compared with control group; # p < 0.05 as compared with MTX group.

Glutathione S transferase activity in the small intestines of control rats and experimental rats with melatonin (20mg & 40mg) treatment, MTX treatment and melatonin pretreatment groups. Data represent mean ± SD, n= 6 (control & melatonin groups); n=8 (MTX & melatonin + MTX groups), * P < 0.05 as compared with 40mg melatonin group; # p < 0.05 as compared with both melatonin groups, $ P < 0.05 compared with 40 mg melatonin group.
**Figure: 6.5.7**

Glutathione reductase activity in the small intestines of control rats and experimental rats with melatonin (20mg & 40mg) treatment, MTX treatment and melatonin pretreatment groups. Data represent mean ± SD, n= 6 (control & melatonin groups); n=8 (MTX & melatonin + MTX groups), * P < 0.01 as compared with control group; # p < 0.05 as compared with MTX group, $ P< 0.05 as compared with melatonin group.

**Figure: 6.5.8**

Myeloperoxidase activity in the small intestines of control rats and experimental rats with melatonin (20mg & 40mg) treatment, MTX treatment and melatonin pretreatment groups. Data represent mean ± SD, n= 6 (control & melatonin groups); n=8 (MTX & melatonin + MTX groups), * P < 0.01 as compared with control and melatonin groups, $ P < 0.05 as compared with melatonin groups.
Figure: 6.5.9

Catalase activity in the small intestines of control rats and experimental rats with melatonin (20mg & 40mg) treatment, MTX treatment and melatonin pretreatment groups. Data represent mean ± SD, n= 6 (control & melatonin groups); n=8 (MTX & melatonin + MTX groups), * P < 0.05 as compared with control group, $ P < 0.05 as compared with Melatonin groups.

Figure: 6.5.10

Superoxide dismutase activity in the small intestines of control rats and experimental rats with melatonin (20mg & 40mg) treatment, MTX treatment and melatonin pretreatment groups. Data represent mean ± SD, n= 6 (control & melatonin groups); n=8 (MTX & melatonin + MTX groups), * P < 0.05 as compared with control group; # p < 0.01 as compared with MTX group; $ P < 0.05 as compared to melatonin groups.
iii) Effect of pretreatment of melatonin on MTX induced nitrosative stress

24 h after the final dose of MTX, the nitrate level was elevated nearly fourfold as compared with control. Pretreatment with melatonin had significantly abolished the MTX – induced increased nitrate levels with both 20mg/kg and 40mg/kg of melatonin (Figure: 6.5.11).

Pretreatment with 20mg melatonin showed decrease in nitrotyrosine staining compared to the MTX group. The decrease in nitrotyrosine staining was confined to the luminal part of the mucosa affecting the apical part of villi. The reduction in nitrotyrosine levels were found to be more in jejunum (Figure: 6.5.12 H) followed by duodenum (Figure: 6.5.12 G) and then ileum (Figure: 6.5.12 I).

With 40mg melatonin pretreatment, nitrotyrosine staining decreased further. Very low levels of nitrotyrosine were seen as small patches in isolated segments of villi epithelium as compared to MTX group. In duodenum (Figure: 6.5.12 J) and jejunum (Figure: 6.5.12 K), the glandular epithelium and the connective cells of the lamina propria were clear of nitrotyrosine staining. In the ileum, nitrotyrosine staining was seen in lamina propria and glandular epithelium (Figure: 6.5.12 L).

In the group that was treated with 20mg of melatonin alone, the nitrotyrosine levels were found to be slightly more compared to the control group, but not as severe as seen in MTX group. Lamina propria / connective tissue cells & glandular epithelium showed moderate expression of nitrotyrosine than seen in villi epithelium. The expression level of nitrotyrosine was highest in ileum (Figure: 6.5.12 O) followed by duodenum (Figure: 6.5.12 M) and then jejunum (Figure: 6.5.12 N) after treatment with 20mg melatonin alone.
In the group that was treated with 40mg of melatonin alone the staining for nitrotyrosine in the small intestines was less compared to the control group and the group treated with 20mg of melatonin alone, except in the ileum where the nitrotyrosine staining was more in the villi, glandular epithelial cells as well as in the connective tissue cells of lamina propria. The expression level of nitrotyrosine is highest in ileum (Figure: 6.5.12 R) followed by duodenum (Figure: 6.5.12 P) and jejunum (Figure: 6.5.12 Q) after treatment with 40mg melatonin.

A six point scale grading of nitrotyrosine staining in duodenum and jejunum is summarized as Vehicle group – 1 (lowest); 40mg melatonin + MTX group – 2; 20mg melatonin + MTX group – 3; MTX – 6 (highest).
Nitrate levels in the small intestines of control rats and experimental rats with melatonin (20mg & 40mg) treatment, MTX treatment and melatonin pretreatment groups. Data represent mean ± SD, n= 6 (control & melatonin groups); n=8 (MTX & melatonin + MTX groups), * P < 0.01 as compared with control group; # p < 0.01 as compared with MTX group, $ P < 0.05 as compared with melatonin groups.
Figure: 6.5.12. Effect of methotrexate on the nitrotyrosine levels in the rat small intestine evaluated by immunohistochemistry. 40X

- (A – C) Mild staining of nitrotyrosine in the control group.
- (D – F) Increased levels of nitrotyrosine were observed in the apical region of the villi as compared to control group. The nitrotyrosine levels were more in ileum (F) followed by duodenum (D) and least in jejunum (E).
Figure: 6.5.12. Effect of pretreatment with melatonin on the nitrotyrosine levels in the rat small intestine evaluated by immunohistochemistry. 40X

(G – I) The levels of nitrotyrosine expression were less that is confined to the luminal part of the mucosa affecting the apical part of villi as compared to the MTX group. The nitrotyrosine levels were more in the ileum (I) followed by duodenum (G) and jejunum (H).

(J – L) Very low levels of nitrotyrosine were seen as small patches in isolated segments of villi epithelium as compared to MTX group. The nitrotyrosine levels were more in the ileum (L) followed by duodenum (J) and jejunum (K).
Figure: 6.5.12. Effect of melatonin on the nitrotyrosine levels in the rat small intestine evaluated by immunohistochemistry. 40X

(M – O) Moderately increased levels of nitrotyrosine were observed in the apical region of the villi as compared to control group. The nitrotyrosine levels were more in ileum (O), followed by duodenum (M) and jejunum (N).

(P – R) Very mild expression of nitrotyrosine levels were seen in the apical region of the villi of duodenum (P) and jejunum (Q) as compared to control group. The nitrotyrosine levels were more in the ileum (R) compared to control and 20mg melatonin group.
The increased expression of PARP in the small intestines by MTX (Figure: 6.5.13 D – F) was found to be decreased when the rats were pretreated with melatonin (Figure: 6.5.13 G – L). Apart from the normal morphology of the cells observed, there was uniform distribution of PARP expression in villi and crypt epithelia as observed in the control group.

In the group that was treated with 20mg melatonin alone (Figure: 6.5.13 M – O), the expression of PARP was found to be more in duodenum and ileum as compared to control group. Muscle and connective tissue cells were also seen with increased PARP expression than seen in vehicle group. The villi and crypts in jejunum showed reduction in intensity generally.

In the 40mg melatonin group (Figure: 6.5.13 P – R), all the three segments of intestine showed reduced immuno expression to PARP as compared with 20 mg melatonin and vehicle treated groups. But, connective tissue cells showed elevated intensity of brown reaction product. Glandular epithelium in all three segments showed very mild expression of PARP than villi epithelium.

A six point scale grading of PARP expression in duodenum, jejunum and ileum is summarized as 40 mg melatonin group – 1 (lowest); Vehicle group – 2; 40mg melatonin + MTX group – 4; 20 mg melatonin + MTX group – 5; MTX – 6.
Figure: 6.5.13. Expression of PARP in response to MTX in the small intestine of rat evaluated by immunohistochemistry. 40X

(A – C) Minimal expression of PARP in the apical region of the villi and crypts in duodenum (A), jejunum (B) and ileum (C).

(D – F) Increased expression of PARP in the apical region of the villi and crypts in duodenum (D), jejunum (E) and ileum (F).
Figure: 6.5.13. Effect of pretreatment with melatonin on the expression of PARP in the rat small intestine evaluated by immunohistochemistry. 40X

(G – I) Decreased expression of PARP with pretreatment of 20mg/kg melatonin as compared to MTX group.

(J – L) Decreased expression of PARP with pretreatment of 40mg/kg melatonin as compared to MTX group.
Figure: 6.5.13. Effect of melatonin on the expression of PARP in the rat small intestine evaluated by immunohistochemistry. 40X

(M – O) Moderately increased expression of PARP was observed in the villi and crypts as compared to control group. The PARP expression was more in ileum (O) followed by duodenum (M) and jejunum (N).

(P – R) Very mild expression of PARP expression was seen in the villi and crypt cells of duodenum (P), jejunum (Q) and ileum (R) as compared to control group.
6.5.4. Discussion

MTX is used extensively in clinical practice for treatment of different types of malignancies and inflammatory disorders. However, MTX administration is often accompanied by side effects and small intestine damage is one of the most commonly seen side effects disturbing the treatment of the patients. Oxygen radicals and hydrogen peroxides are linked with the adverse effects of anti-tumor drugs (551). Our study had shown MTX – induced oxidative and nitrosative damage. Compared to other antioxidants, melatonin has greater efficacy in protecting against cellular oxidative stress and it can preserve macromolecules including DNA, protein and lipid from oxidative damage (552). Melatonin treatment was shown to increase villus height, total mucosal thickness, and crypt cell mitosis after bowel resection in rats (553). Considering the potential of melatonin as an antioxidant, it was hypothesized that melatonin would protect the small intestine from the oxidative and nitrosative stress and thus can minimize the morphological features of cell damage associated with MTX administration.

The results of the current study indicate that melatonin at a concentration of 40mg/kg reduced the MTX – damage of the small intestine as evidenced by the recovery of the villi and decrease in the transmural acute inflammatory infiltrate of the mucosa and the muscularis propria. The protective effect of melatonin on the architecture of the small intestine was reported by others (554, 555). Pretreatment with melatonin had decreased the MTX – induced oxidative stress as evidenced by decrease in the malondialdehyde levels following melatonin pretreatment. The protective role of melatonin against oxidative stress induced by various methods in the small intestines of rats has been demonstrated earlier (554, 556-558). Melatonin has been shown to reduce lipid peroxidation in non – septic shock induced by zymosan (559). Melatonin has been
shown to ameliorate lipid peroxidation in cerulean – induced acute pancreatitis (560) as well as in intestinal ischemia/reperfusion injury in rats (561).

Besides acting as a direct scavenger of free radicals melatonin has indirect antioxidant actions. Melatonin is known to stimulate the activities of the antioxidant enzymes including SOD, Glutathione reductase, glutathione peroxidase, and catalase (562-565). Accordingly, in this study, melatonin pretreatment had restored the activities of glutathione peroxidase, glutathione reductase, SOD and catalase, but had no effect on the activities of glutathione S transferase and MPO. These results suggest that melatonin by virtue of its antioxidant property had decreased the oxidative damage caused by methotrexate but could not prevent the associated neutrophil infiltration and inflammation. In this study, pretreatment with melatonin had attenuated MTX induced oxidative stress, nitrosative stress and small intestinal damage.

Melatonin had also abolished MTX – induced nitrosative stress as evidenced by the decreased nitrate levels, nitrotyrosine levels and PARP expression in the small intestine of melatonin – MTX treated rats. Melatonin being a scavenger of free radicals protects the intestine from the reactive nitrogen species. Earlier study had shown the protective effect of melatonin on increased nitrotyrosine levels and PARP activation in an acute model of inflammation (566), and also to inhibit apoptosis through the inhibition of PARP activation (567, 568).

A number of studies have shown that melatonin is significantly better than the classic antioxidants in resisting free radical based molecular destruction. Melatonin was known to be more effective than vitamin E, β carotene, vitamin C, and superior to garlic oil (569). An important advantage of melatonin over classical antioxidants is its lack of prooxidative actions. All classical antioxidants are potential electron donors and they exhibit both reduced and oxidized forms. Once they donate an electron to neutralize free radical, they are transformed
from a reduced to an oxidized state. Usually, the oxidized form will be regenerated to the reduced state through the mechanism known as redox reaction or recycling. In this pathway, the recycling of vitamin C or vitamin E occurs at the expense of GSH. Because these antioxidants are electron donors and exhibit redox reactions, their oxidized forms also can oxidize other molecules. Therefore, classical antioxidants are prooxidants. Melatonin sacrifices itself and does not participate in redox cycling after scavenging free radicals. Thus, melatonin is classified as a suicidal or terminal antioxidant.

Melatonin has been administered in both physiological and pharmacological amounts to humans and animals; and there is widespread agreement that it is a non–toxic molecule (570). In pregnant rats, doses up to 200 mg/kg/day were found to be non–toxic (571). Melatonin is easily synthesized in a pharmacologically pure form and is inexpensive and affordable.

Beneficial antioxidant effects of melatonin have been recently shown in clinical settings for several chronic diseases, including patients with rheumatoid arthritis (572), elderly patients with primary essential hypertension (573), and females with infertility (574).

Thus, the current study shows that MTX–induced small intestinal damage can be reduced by the prior administration of melatonin. Therefore, it is suggested that melatonin may be beneficial in minimizing MTX induced small intestinal damage in humans. However, it should be borne in mind that pharmacological doses and not physiological dose of melatonin protects against oxidative stress, nitrosative stress and organ injury. Melatonin plays an important role in the regulation of various body functions including circadian rhythms, blood pressure, seasonal reproduction, and immunity. Therefore, before administering melatonin to humans the side effects of long term intake of melatonin needs to be verified.