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Effect of methotrexate treatment on iNOS gene expression, protein tyrosine nitration protein cysteine nitrosylation, and the activities of mitochondrial enzymes in the small intestinal mucosa of rats

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ABSTRACT

Aim: Methotrexate (MTX) is a structural analogue of folic acid and is widely used in the treatment of leukaemia and other malignancies, rheumatoid arthritis and other rheumatic disorders. Gastrointestinal mucositis is a frequent dose-limiting side effect of methotrexate (MTX) chemotherapy. The underlying mechanisms of the mucositis is not completely understood. In the present study we examined the effect of MTX treatment on iNOS gene expression, protein tyrosine nitration protein cysteine nitrosylation, and the activities of mitochondrial enzymes in the small intestinal mucosa of rats Methods: Gastrointestinal injury was induced in the rats by the administration of 3 consecutive i.p. injections of 7 mg /kg body wt. MTX .The small intestines were used for light microscopy and immunohistochemical localisation of iNOS, nitrotyrosine (NTy) and nitrocysteine (NCy). Mucosal scrapings were used for iNOS mRNA expression by RTPCR, iNOS protein, nitrotyrosine and protein cysteine adducts formation by western blot and assay of key mitochondrial enzymes. Results: MTX treatment resulted in moderate to severe damage to the small intestines. iNOS mRNA expression , iNOS protein, NTy protein adduct and NCy adducts were increased in the MTX treated rat intestines. The activities of electron transport chain (ETC) complexes IV and V were decreased by 66% and 71 % respectively in the MTX treated rat intestines. With respect to tricarboxylic acid (TCA) cycle enzymes, aconitase activity was decreased by 73 %, succinate dehydrogenase by 85 % and glutamate dehydrogenase by 61%. With respect to antioxidant enzymes, superoxide dismutase (SOD) activity was increased by 40 %, and that of catalase, glutathione reductase, were decreased by 68 % and 66%, respectively. Conclusion: The gastrointestinal toxicity induced by MTX may be due to increased nitrosative stress. The decreased activities of the mitochondrial energy producing enzymes and antioxidant enzyme may be due to their inactivation by MTX induced nitric oxide overproduction

Key words: Methotrexate; iNOS; gastrointestinal mucositis; protein tyrosine nitration; protein S nitrosylation; ETC complexes; mitochondrial antioxidant ezymes.

Introduction

Methotrexate (MTX) is a structural analogue of folic acid and is widely used in the treatment of leukaemia and other malignancies. It is currently the most common anti-rheumatic drugs prescribed for the treatment of rheumatoid arthritis and other rheumatic disorders [1]. One of the major toxic effects of MTX is intestinal injury and enterocolitis [2, 3]. Since the mechanism of gastrointestinal toxicity of MTX is not completely known, cancer chemotherapy has to be accompanied by symptomatic therapy such as antibiotics and anti-diarrheal drugs. It is important to unravel the mechanism by which MTX induces intestinal damage in order to prevent this side effect and hence perform effective cancer chemotherapy.

Evidence suggests that overproduction of NO by iNOS has deleterious effects on the GIT [4]. Nitric oxide has short half-life and immediately reacts with

the superoxide anion to form the peroxynitrite [5]. Because peroxynitrite is a transient species with a biological half-life even shorter than that of nitric oxide, it immediately nitrates specific tyrosine residues on proteins to produce 3-nitrotyrosine (3-NT) 5,6[11, 16. NO and peroxynitrite could interact with a wider variety of biological targets, with two leading mechanisms involving 1) Snitrosylation of cysteine, and 2) nitration of tyrosine residues comprised within a variety of proteins [6,7]. In particular, PON is a central contributor to protein nitration and its major product 3-nitrotyrosine (3-NT) is widely used as the footprint for PN formation [6]. Nitration of tyrosine residues may lead to loss of protein structure and function [8,9].Some of the PON target mitochondrial proteins are key enzymes in energy production; these include enzymes that are involved directly or indirectly in the citric acid cycle (e.g. aconitase, succinate dehydrogenase, glutamate dehydrogenase) , enzymes of the electron transport chain (e.g., complexes I, II, complex IV (cytochrome oxidase) and Complex V(F1 ATPase) and energy distribution (e.g., creatine kinase). In addition to these mitochondrial proteins, five enzymes that protect cells against oxidative damage appear to be nitrated: MnSOD, catalase, glutathione *S*-transferase, glutathione reductase and carbonic anhydrase III [10].

It is known that the rat is a suitable model for the study of pathogenesis of gastrointestinal toxicity of chemotherapeutic agents [11].Therefore we assessed the effect of MTX on iNOS protein and mRNA expression by western blot and RT PCR respectively. Nitrotyrosine (NTy) and nitrocysteine (NCy) production were assessed by immunohistochemistry and western blot. The activities of mitochondrial energy producing enzymes and antioxidant that are known to undergo nitration and inactivation in inflammatory conditions were also assayed in the small intestines of rats.

Materials and methods:

Chemicals

Methotrexate, secondary antibody to anti-tyrosine antibody, primary monoclonal anti-nitrotyrosine antibody were obtained from Santa Cruz (Bombay, India) and the Super Sensitive Polymer/HRP/DAB kit was obtained from BioGenex (Chennai, India). All other chemicals used were of the highest analytical grade available.

Animals

Adult male Wistar rats (150-200 gm) were used for the study. They were housed in standard rat cages. All the rats were exposed to 12 hour light-dark cycles and allowed access ad libitum to water and rat chow. All experiments were carried out in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and the Institutional Animal Ethics Committee (IAEC) approval (CPCSEA/IAEC/14/2009/, dated October 9, 2009). All the guidelines were strictly adhered to and sufficient measures were adopted to minimize pain and discomfort with animal experimental procedures.

Animal treatment

Methotrexate was administered to the rats at the concentration of 7 mg/kg body weight intraperitoneally for three consecutive days as described in the literature causing consistent intestinal injury in normal rats [12]. Control animals received an equal volume of the vehicle alone for three consecutive days intraperitoneally.

Tissue procurement

On the fourth day, the MTX treated rats and control rats were anesthetized with halothane and killed by cervical dislocation after overnight fast. The abdomen of each rat was opened and the entire length of the small intestine was removed, washed with ice cold normal saline and used for studies. A piece of small intestine (duodenum, jejunum and ileum) was stored for histology and histochemistry studies. The rest of the intestine was cut opened along its anti-mesenteric border longitudinally and mucosa was harvested by gently scraping with a glass slide from the remaining tissue and stored for biochemical studies.

Preparation of intestinal homogenate

A 10% homogenate of the mucosal scrapings was prepared in homogenizing buffer containing 250 mM sucrose, 5mM HEPES, 1mM EGTA, 0.5mg/ml BSA (pH 7.4). The homogenate was centrifuged at 1000g for 10min at 4^{o}C and the supernatant was used for the biochemical assays.

Biochemical assays

Assay of the activities of the ETC Complexes

The activities of complexes IV and V were determined as described earlier [13,14]. The

extinction coefficient for NADH is 6.22. The enzyme activities is expressed as mol/min/mg protein.

Succinate Dehydrogenase Activity

The activity of succinate dehydrogenase was assayed using INT as an electron acceptor, which forms formazan crystals on reduction [13]. The molar extinction co-efficient of formazan in ethyl acetate is 20.1×10^3 at *A*490 nm. One unit of enzyme is defined as the amount required for converting 1 μmol of INT to its formazan per minute under standard assay conditions.

Assay of other mitochondrial enzymes

The activity of aconitase was measured as a rate of NADP reduction by isocitrate dehydrogenase [15]. The change in absorbance was measured at 340nm. Aconitase activity was calculated with molar extinction co – efficient of $NADP^+$ is 6.22 x 10^3 at A340 nm and expressed as unit/mg protein. The activity of glutamate dehydrogenase was measured in the direction of α-ketoglutarate amination [16]. The change in absorbance was measured at 340nm and the glutamate dehydrogenase activity was calculated with molar extinction co – efficient of $NADP^+$ is 6.22 x 10^3 at A340 nm and expressed as unit/mg protein. Creatine kinase activity was measured at the reduction rate of NADP by glucose-6-phosphate dehydrogenase [17]. The increase in absorbance was measured at 340nm. Creatine kinase activity was calculated with molar extinction co – efficient of $NADP^+$ is 6.22 x 10^3 at A340 nm and expressed as unit/mg protein.

Assay of antioxidant enzymes

Superoxide dismutase was measured as described by Ohkuma et al [18]. Amount of superoxide formed was calculated using the molar extinction coefficient of MTT formazan E540 of 17,000 M^{-1} cm^{-1} at pH 7.4. Catalase activity was estimated by measuring the change in absorption at 240 nm using H_2O_2 as substrate [18]. For the assay of glutathione reductase the rate of oxidation of NADPH in the presence of GSSG was measured at 340 nm [18]. Proteins present in the sample was estimated by Lowry's method [19].

Light microscopy

The small intestine segments (duodenum, jejunum, ileum) was fixed in 10% buffered formaldehyde, dehydrated in graded ethanol and embedded in paraffin. Five-micron sections were cut on a microtome, mounted on clear glass slides and stained with haematoxylin and eosin. The sections were examined by light microscopy (Leica) and evaluated in a blinded fashion. Mucosal injury, inflammation and hyperemia/hemorrhage was assessed and graded in a blinded manner using the histological injury scale previously defined by Chiu et al [20].

Immunohistochemistry for iNOS, NTy and NCy

The small intestine segments (duodenum, jejunum, ileum) was fixed in 10% neutral formalin and five micron sections on PLL coated slides were obtained from paraffin-embedded tissues. After deparaffinization, the sections were permeabilized for antigen retrieval with 0.1% Triton X-100 in Tris-buffered saline for 30min. Endogenous peroxidases were quenched by 3% hydrogen peroxide for 15 min. After the buffer wash, the universal protein blocking agent was applied and incubated for 15min. Then, the respective primary antibody for iNOS, NTy, and NCy(1:100) was applied over the sections and incubated overnight followed by super enhancer for 30min. The bound primary antibody was detected by the addition of secondary antibody conjugated with horseradish peroxidase polymer and DAB substrate. Later the slides were counterstained with Harris Haematoxylin and mounted with DPX mountant medium. Sections were examined under bright field light microscopy (Olympus - cellsens standard software) and evaluated. Results were the average of counting three sections from each rat, with five rats per condition, and were expressed as percent of immunopositive cells compared to total number of cells. The staining was scored as described by Young et al. [21].Score 0 -positive cells <10%, 1+ 10-25%, 2+ 25-50%, 3+50-75% and 4+ >75%.

Western Blotting for nitrotyrosine (NTy) and nitrocysteine (NCy)

The mucosal scrapings was homogenized using lysis buffer containing 250mM sucrose, 20mM HEPES, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, (pH – 7.5) and added 1mM dithiothreitol, 1mM PMSF, protease inhibitor cocktail on day of experiment. The 10% homogenate prepared was centrifuged at 1000g for 10min at 4°C and used for the estimation of NTy and NCy. Protein concentration was determined by Lowry's method [19]. Samples containing 50-100 µg protein were denatured and separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), along with ECL DualVue western blotting molecular weight markers (Amersham). Proteins

were transferred to 0.45µm polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk or bovine serum albumin (BSA) in TBS and 0.1% Tween-20 for 1hr. Thereafter, the membranes were incubated over-night in a cold room with NTy and NCy antibodies. After incubation with the respective primary antibodies, anti-mouse or anti-rabbit secondary antibodies conjugated with horse-radish peroxidase were used to detect bound primary antibodies. Visualization of the bands of interest was carried out by use of a chemiluminescent substrate. The developed bands were visualized and documented using the AlphaEase FC gel documentation system (Alpha Innotech Corporation, CA.)

iNOS mRNA expression by RT PCR

Total RNA was isolated from the snap-frozen small intestinal mucosal scrapings obtained from experimental animals using TRI-reagent. RNA obtained was reverse-transcribed to cDNA. This was done using 1 µg of total RNA, using a reverse transcriptase core kit, according to the manufacturer 's instructions (Eurogentec, Belgium). Levels of expression of iNOS mRNA were quantitated using the qPCR MasterMix Plus for SYBR green I dNTP kit (Eurogentec, Belgium), according to the manufacturer's instructions. The expression of iNOS gene was calculated using ΔΔct method. Quantitative measurements of each gene were derived from a standard curve constructed from known concentrations of PCR product. The expression of iNOS gene of interest was calculated relative to that of β-actin, which was used as a house keeping gene.

Primer sequences used for PCR were given below:

iNOS Forward: 5'- AGG-TGT-TCA-GCG-TGC-TCC-AC -3'

Reverse: 5'- AGT-TCA-GCT-TGG-CGG-CCA-CC -3'

β actin Forward : 5'- CCT-CTA-TGC-CAA-CAC-AGT-GC -3'

Reverse: 5'- ACA-TCT-GCT-GGA-AGG-TGG-AC -3'

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software package, version 16. All parameters were studied in a minimum of 6 animals for statistical validity. The data represent the mean value ± SD and analyzed by Mann-Whitney test. A value of $P < 0.05$ was taken to indicate statistical significance.

Result:

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

Under light microscope, the duodenum, jejunum and ileum of the control group rats showed normal architecture (Fig.1 A – C).After the final dose of MTX, there was moderate destruction of the villi and the crypts of Lieberkühn in duodenum (Fig.1 D). In the jejunum, there was more destruction of the villi. The villi were atrophied and focally absent (Fig.1 E). In the ileum, the villi were atrophied, blunted and fused. There was destruction of the crypts of Lieberkühn (Fig.1F). Based on the light microscopic changes the intestine sections from the two groups were graded on a 5 point scale previously defined by Chiu et al [36]. The small intestine sections in control group revealed normal morphology with normal villi and crypt cells (0 or Grade 1). The duodenum and jejunum sections in MTX group showed atrophied villi with exposed lamina propria and there was acute inflammatory infiltrate (Grade 4). The ileum sections from the MTX group revealed fused villi, digestion and disintegration of the lamina propria in villi and presence of hemorrhage and ulceration (Grade 5). The damage was comparatively more in the ileum (grade 5), followed by jejunum and duodenum (grade 4).

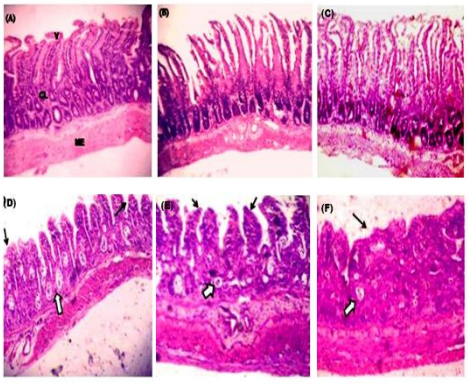

Fig 1. Representative figures of the duodenum (A), jejunum (B) and ileum (C) of control rats showing normal architecture, magnification X100.V – Villus, CL – Crypts of Leiberkhun; ME – Muscularis Externa. Representative figures of small intestine of experimental rats after treatment with MTX, magnification X100. Black arrow indicates the damaged villi and the white arrow indicates the crypt abscess. The villi were shortened in the duodenum (D), distorted in the jejunum (E) and aborted, flattened, blunted and fused in the ileum (F).

MTX treatment resulted in increased iNOS protein and mRNA expression

Immunohistochemical analysis of the control rat intestines showed weak immunostaining for iNOS in the villus and crypt regions as iNOS is only expressed in the inflammatory conditions (Fig.2 A). MTX-treated rats presented intense iNOS immunostaining in the enterocytes, lamina propria cells, and neutrophils and other inflammatory cells surrounding and within necrotic crypts (Fig.2B). Immunohistochemical (IHC) grading for iNOS protein is control +/-, duodenum and jejunum 2+, and ileum 3+.These results suggest that inducible NOS is constitutively expressed in enterocytes, and is induced in the rat intestines after MTX treatment. Our findings were confirmed by quantification of iNOS protein by western blot analysis which revealed 6 fold increase in iNOS protein level in the small intestines of MTX treated rat (fig.3).

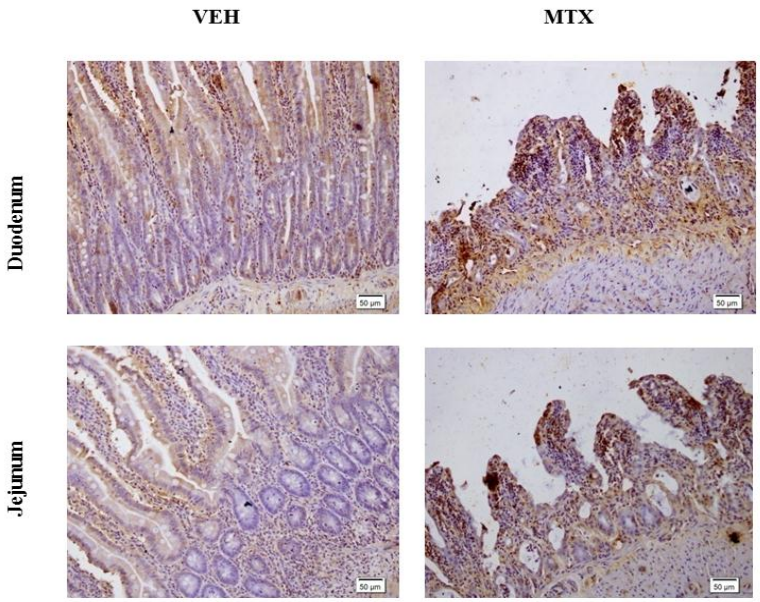

Fig.2: Representative immunohistochemical images of inducible nitric oxide synthase (iNOS) protein in the small intestine segments of control rats and MTX treated rats (20X). Control rats showed minimal basal immunostaining for iNOS, while MTX treated rats shown intense immunostaining of iNOS in the duodenum and jejunum when compared to control.

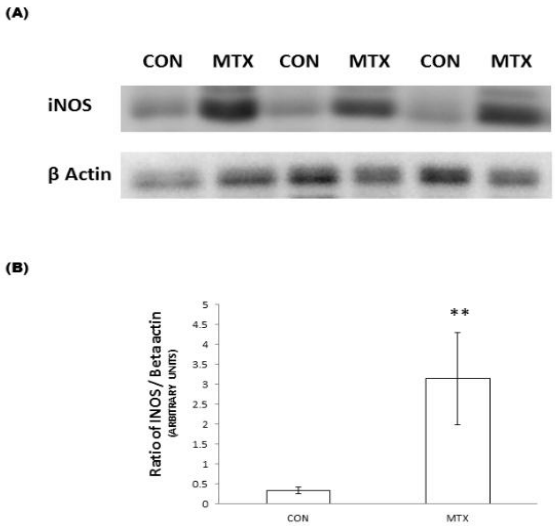

Fig.3:A. Representative Western blots of iNOS protein levels in the small intestines of control rats and MTX treated rats. B. iNOS protein quantification by scanning densitometry. Data represent mean ± SD, N = 4 in each group, ** P value < 0.01 as compared to control.

The effect of MTX on iNOS transcription and mRNA expression was analysed by reverse transcriptase polymerase chain reaction. Untreated rat intestines contained detectable amount of iNOS mRNA suggesting its basal expression. MTX treatment resulted in more than 2 fold increase in iNOS mRNA as compared to control (Fig. 4). These findings suggest role for iNOS induction in MTX induced small intestinal injury.

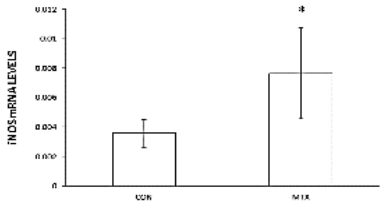

Fig.4: Effect of MTX on iNOS mRNA levels. RT-PCR analysis of iNOS mRNA expression in the mucosal scrapings of control rats and MTX treated rats. Data represent mean ± SD, N = 6 in each group, * P value < 0.05 as compared to control.

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

Weak and uniform basal immunostaining for NTy was found in the small intestine segments of control rats. MTX treated rats showed intense immunostaining for NTy expression in enterocyte regions, lamina propria cells of small intestine segments (duodenum, jejunum, ileum) (fig.5) . IHC grading for NTy adduct is control +/-, duodenum and jejunum 2+, and ileum 3+.

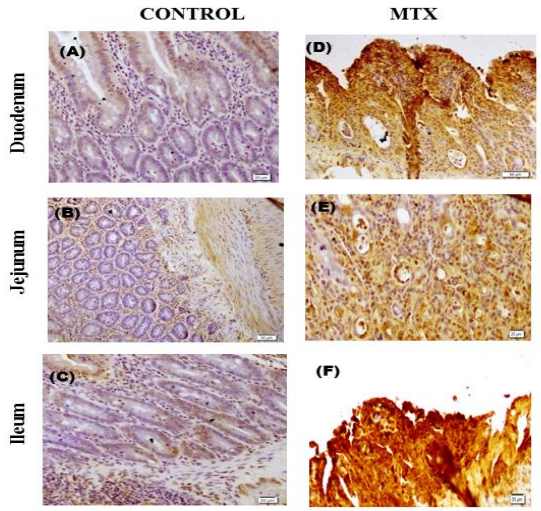

Fig. 5: Representative image of nitrotyrosine (NTy) protein expression in the small intestine segments of control rats and MTX treated rats (20X). Negligible NTy immunostaining was observed in the control rat (A-C). MTX treated rats showed intense immunostaining for NTy in all the segments of the small intestines, ileum>duodenum=jejunum

With regard to NCy, moderate staining was observed in the small intestines of control rats suggesting that cysteine nitrosylation is a physiological process in the small intestines. In the MTX treated rat intestines the staining for NCy was more intense as compared with control (fig. 6). IHC grading for NCy in control duodenum is 1+, jejunum and ileum 2+ and in MTX treated rats 3+ for all the segments of the small intestines.

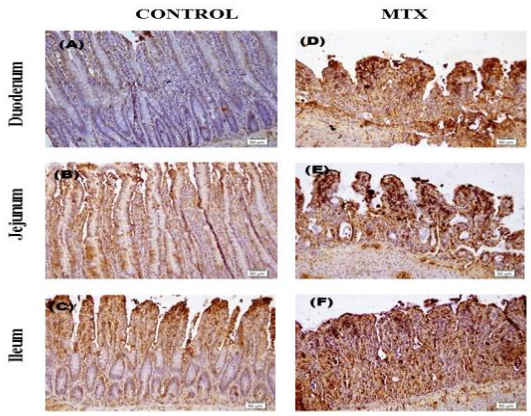

Fig.6: Representative image of nitrocysteine (NCy) protein expression in the small intestine segments of control rats and MTX treated rats (20X). Moderate basal NCy immunostaining was observed in the control rat (A-C). MTX treated rats showed intense immunostaining for NCy in all the segments of the small intestines (D-F).

In order to confirm the formation of 3NT adducts, we carried out western blots in whole intestinal homogenates and probed with NT antibody. 3 Nitrotyrosine adducts were present in detectable amounts in the small intestines of control rats. The small intestinal homogenates from the MTX treated rats had higher nitrotyrosine protein adducts as compared with the controls (fig. 7). This clearly demonstrates that there was some intrinsic nitration in some proteins in the normal intestine, and the protein nitration was significantly enhanced after MTX injury. Overall these results suggest that there is overproduction of PON, increased protein tyrosine nitration and cysteine nitrosylation in the small intestines of MTX treated rats.

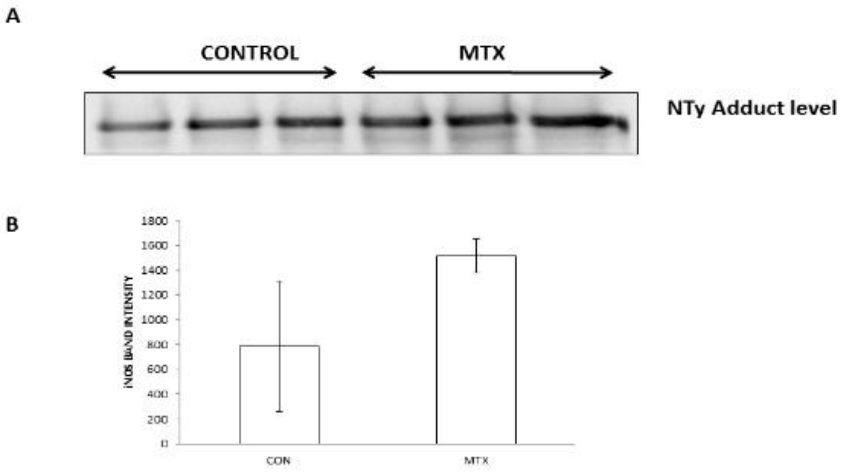

Fig.7A. Representative immunoblot of nitrotyrosine protein levels in the small intestines of control rats and MTX treated rats. B. Quantification of NTy proteins in the small intestines by scanning densitometry. Data represent mean ± SD, n = 4 in each group

MTX treatment decreased the activities of ETC complexes The activities of mitochondrial ETC complexes were decreased significantly, complex IV by 66% (Fig.8A), and complex V (F1ATPase) by 71 % (Fig.8B) in the MTX treated rat intestine as compared with control.

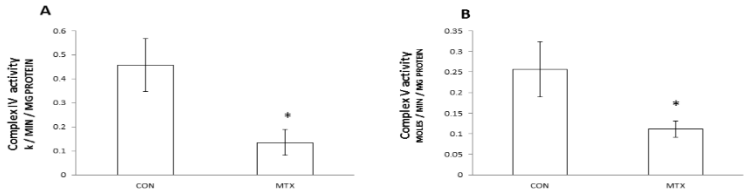

Fig.8:A. Effect of MTX treatment on complex IV (cytochrome-c-oxidase) activity in the small intestines of rats. Data represent mean ± SD, n = 6 in each group, * P value < 0.02 as compared to control. B. Effect of MTX on complex V (ATPase) activity in the small intestines of rats. Data represent mean ± SD, n = 6 in each group, * P value < 0.05 as compared to control.

MTX decreased the activities of TCA cycle enzymes and creatine kinase

The activities of TCA cycle enzyme aconitase was reduced by 73 % (Fig.9A) and succinate dehydrogenase by 85% (Fig.9B) in the small intestines of MTX treated rats as compared with control. The activity of glutamate dehydrogenase, marker of mitochondrial injury was decreased by 61 % (Fig. 9C) and creatine kinase was decreased by 71% in the small intestines of MTX treated rat intestines as compared with control (Fig.9D).

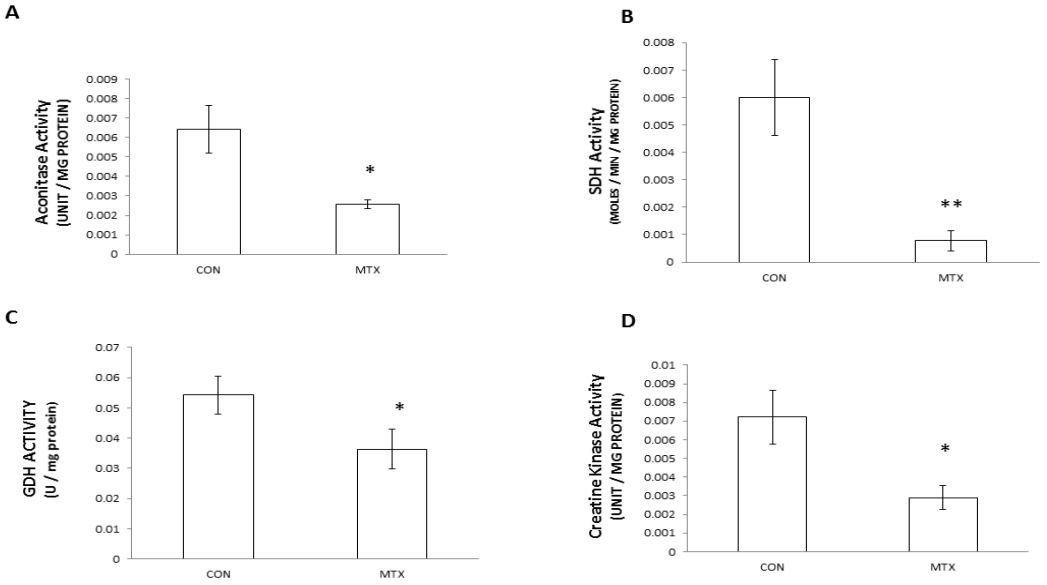

Fig.9. A. Effect of MTX treatment on aconitase activity in the small intestines of rats. Data represent mean ± SD, n = 6 in each group, * P value < 0.05 as compared to control. B. Effect of MTX treatment on succinate dehydrogenase activity in the small intestines of rats. Data represent mean ± SD, n = 6 in each group, ** P value < 0.02 as compared to control. C. Effect of MTX treatment on glutamate dehydrogenase activity in the small intestines of rats. Data represent mean ± SD, n = 6 in each group, * P value < 0.05 as compared to control. D. Effect of MTX treatment on creatine kinase activity in the small intestines of rats. Data represent mean ± SD, n = 6 in each group, * P value < 0.05 as compared to control.

MTX altered the antioxidant enzyme activity

To our surprise, the activity of superoxide dismutase, the enzymes that has been shown to undergo tyrosine nitration and inactivation was increased 40% in the small intestines of MTX treated rats as compared with control (Fig.10A). With respect to the activities of other antioxidant enzymes, catalase activity was decreased by 68 % (Fig.10B), glutathione reductase was decreased by 66% (Fig.10C)

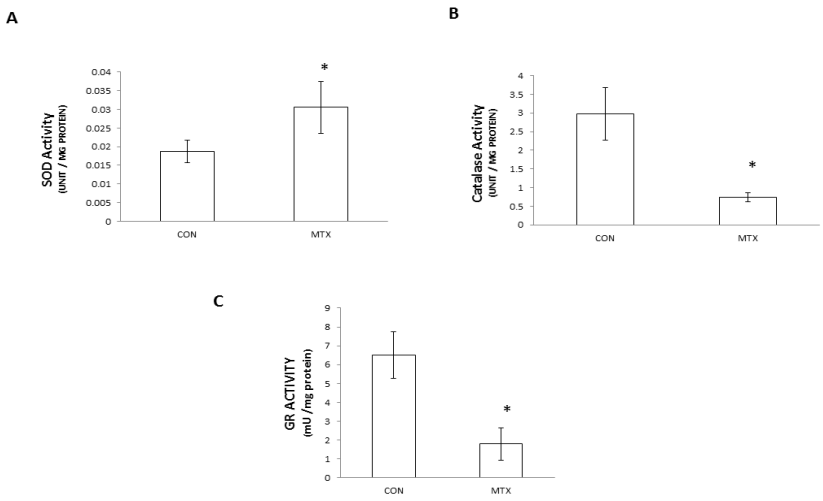

Fig. 10. A. Effect of MTX treatment on SOD activity in the small intestines of rats. Data represent mean ± SD, n = 6 in each group, * P value < 0.05 as compared to control. B. Effect of MTX treatment on catalase activity in the small intestines of rats. Data represent mean ± SD, n = 6 in each group, * P value < 0.05 as compared to control. C. Effect of MTX treatment on glutathione reductase activity in the small intestines of rats. Data represent mean ± SD, n = 6 in each group, * P value < 0.01 as compared to control.

Discussion

MTX is a well-known cause of intestinal mucositis, which impairs rapidly dividing cells, such as epithelial stem cells within intestinal crypts, thereby causing diminished enterocyte replacement. Since the mechanism of gastrointestinal toxicity of MTX is not completely known, cancer chemotherapy has to be accompanied by symptomatic therapy such as antibiotics and anti-diarrheal drugs. In our previous study we have demonstrated that overproduction of NO contribute to MTX enteritis [22]. We hypothesised that overproduction of NO may play a vital role in MTX enteritis.

Studies have demonstrated that sustained release of NO as a result of iNOS upregulation may lead to cellular injury and gut barrier failure [23]. Accordingly in the present study we observed a 2 fold increase in iNOS mRNA and protein expression in the small intestines of MTX treated rats. Our findings are in agreement with those reported earlier by Leitão et al [24]. During inflammatory reactions when large amounts of NO and superoxide are formed, the combination of both leads to the formation of reactive nitrogen species, such as the peroxynitrite. Peroxynitrite is a central contributor to protein nitration and its major product 3-nitrotyrosine (3-NT) is widely used as the footprint for PON formation. Immunohistochemistry is an important and often used tool in the investigation of nitrotyrosine. Therefore we analysed 3-NT in the intestinal tissues by immunohistochemical methods and as well as western blot. Basal levels of 3NT were detectable in the small intestines of control rats suggesting a physiological role for protein tyrosine nitration. A significant increase in nitrotyrosine immunostaining was observed in the intestinal segments of MTX-treated rats. In addition we were able to demonstrate using western blot technique more than 2 fold increase in 3NT adducts in MTX treated rat intestine as compared with control, reinforcing the role of NO via peroxynitrite in intestinal mucositis.

PON can interact with cysteine residues of proteins resulting in S nitrosylation of cysteine residues comprised within a variety of proteins. We observed moderate basal expression of NCy in the small intestines of controls rat, suggesting a physiological role for this process. We observed significant increase in NCy in the small intestines of MTX treated rats as compared with control. Protein S-nitrosylation has been demonstrated to be a key modification of cysteine residues under a variety of physiological and pathophysiological conditions [25]. There are data suggesting that S-nitrosylated thiols are protected from irreversible oxidation [26]. In particular, in connection with nitric oxide-based redox regulation of protein function, S-nitrosylation has been found to be involved in protective mechanisms in many disorders [25].The present study is the first one to demonstrate increased protein cysteine nitrosylation in the small intestines of MTX treated rats. Increased protein S nitrosylation observed in the MTX treated rat

intestines may be a defense mechanism to protect the protein thiols from irreversible oxidation.

During inflammatory conditions, PON has been shown to selectively nitrate some proteins and inactivate them [27-29].Some of the PON target proteins are mitochondrial proteins that are key enzymes in energy production; these include enzymes that are involved directly or indirectly in the citric acid cycle (e.g., glutamate dehydrogenase, aconitase, and succinate dehydrogenase) and are involved in the electron transport chain (e.g., complex II, cytochrome oxidase (complex IV), and complex V (F1 ATPase) and energy distribution (e.g. creatine kinase) [29].Therefore, we assayed the activities of these enzymes in mucosal homogenates.

With respect to ETC complexes, the activities of Complexes IV and V were lowered by 66% and 71% respectively in the small intestines of MTX treated rat. Complex IV is the primary site of cellular oxygen consumption and is thus central to oxidative phosphorylation and the generation of ATP. A decrease in its activity has been shown to reduce ATP production [30].Studies have shown that excess NO inhibits cytochrome-c oxidase and superoxide may transiently leaks from the ETC, leading to the formation of peroxynitrite [31].The activity of Complex V (ATP synthase) was decreased by 71% in MTX treated small intestines as compared with control. Mitochondrial ATP synthase is essential for providing cellular energy (i.e., ATP) for proper maintenance and survival of all living cells. If ATP synthase (mitochondrial complex V) is inhibited, this leads to depletion of an essential energy source, thus contributing to necrotic tissue injury.

We next assessed the activities of enzymes that are reported to undergo nitration during inflammatory conditions, and are directly or indirectly related to energy production namely aconitase, succinate dehydrogenase (SDH), glutamate dehydrogenase(GDH) and creatine kinase (CK) in the small intestines of control rats and MTX treated rats. We found a significant decrease in aconitase activity in the MTX treated rat intestines as compared with controls. Aconitase, the TCA (tricarboxylic acid) cycle enzyme participates in mitochondrial energy production and cellular iron regulation [32] Decrease in aconitase activity is likely to affect the overall turnover efficiency of the citric acid cycle thereby decreasing energy production. In addition, iron released from aconitase can further propagate intramitochondrial oxidative damage by metal-mediated formation of oxidizing and nitrating species.

With respect to SDH activity, one of the most important marker enzymes for mitochondria, an 85 % decrease was observed in MTX treated rat intestines as compared with control. SDH is the marker of mitochondrial inner membrane integrity, and its activity shows the degree of mitochondrial activity [33]. A reduction in SDH activity has been reported to decrease the rates of mitochondrial respiration and ATP production [33].

Glutamate dehydrogenase (GDH) activity was decreased by 61% in MTX treated rat intestines as compared with control. GDH, located in the mitochondrial matrix, catalyzes the reversible deamination of glutamate to α-ketoglutarate, ammonium, and NADH. This α-ketoglutarate (KG)can be channelled into the Kreb's cycle to stimulate ATP production [34].Thus decrease in GDH activity can decrease the availabiliity of KG for energy production

We also assayed the activity CK, an enzyme which plays a key role in energy homeostasis. We found 70% decrease in the activity in the MTX treated rat intestines as compared with control. Reduced activity of CK can reduce ADP supply to the mitochondrial ATP synthase and thereby compromise energy generation [35]. CK is of importance in the small intestines, where energy demands is high due to absorption of nutrients and continual enterocyte renewal.

In addition to these mitochondrial proteins, five enzymes that protect cells against oxidative damage appear to be nitrated: MnSOD, catalase, glutathione *S*-transferase, glutathione reductase and carbonic anhydrase III. Therefore we assayed MnSOD, catalase and glutathione reductase in the intestinal mucosa.

With respect to SOD activity surprisingly we found an increase in activity in the MTX treated rat intestines. Most of the studies have given rise to a reduced activity of SOD [36,37]. However, in our study, we found significant increase in MnSOD activity in the intestines of MTX treated rats as compared to control. This may be an adaptive mechanism by the body to combat excessive free radicals that are produced.

Catalase activity was significantly decreased in the small intestines of MTX treated rats. Catalase is one of the well-known antioxidant enzymes and detoxifies hydrogen peroxide. It is widely expressed in the cytoplasm and peroxisomes of gastrointestinal epithelium and lamina propria and is activated when concentrations of H2O2 increase, e.g. during inflammatory process [38]. The decreased activity of catalase may result in accumulation of H_2O_2contributing MTX induced increased oxidative stress and small intestinal injury.

The activity of glutathione reductase (GR), the enzyme crucial for the regeneration of reduced glutathione was significantly decreased in the small intestines of MTX treated rats as compared with 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 to increased oxidative stress and tissue injury.

In the present study MTX treatment resulted in iNOS gene induction and increased iNOS protein, PON overproduction (as evidenced by 3NT), and protein S nitrosylation in the small intestines of rats suggesting MTX induced small intestinal injury may be mediated via increased nitrosative stress. 3NT accumulation was accompanied by decreased activities of enzymes that are known to undergo nitration and inactivation by peroxynitrite in *in vivo* inflammatory conditions. These enzymes include mitochondrial enzymes required for ATP synthesis including complexes IV, V, aconitase, SDH and GDH. Enterocytes have a high requirement of ATP for absorption of nutrients and self renewal. We propose that energy depletion due to inactivation of the ETC complexes and vital TCA cycle enzymes, aconitase, SDH and GDH may limit enterocyte function and renewal, resulting in gastrointestinal injury

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