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Efficacy of Variable Dose of Aspirin to Combat Methotrexate Induced Intestinal Toxicity in Albino Rats

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Abstract

The aim of the present study was to particularize the effect of variable dose of aspirin against intestinal toxicity in animals. The albino rats were indiscriminately divided into six groups and subjected to thirteen weeks treatment with sham control (3ml/kg p.o, normal saline); toxic control (2.5 ml/kg i.p, MTX**);** low dose of aspirin (8 mg/kg, p.o); low dose aspirin and MTX (8 mg/kg, p.o + 2.5 ml/kg, i.p), high dose aspirin (45 mg/kg, p.o); high dose aspirin and MTX (45mg/kg, p.o + 2.5 ml/kg, i.p). Intestinal toxicity of aspirin was appraised on the basis of biochemical changes and modulation in the inflammatory markers. Low dose of aspirin flaunted sententious protection against the MTX induced toxicity. Whereas, the high dose of aspirin was observed to hasten the same. The high dose of aspirin has also demonstrated altered biochemical changes in physiological system but not in resemblance to the low dose when given alone.

Key words: Aspirin, Interleukin, Intestinal toxicity, Methotrexate, NSAIDS, Oxidative stress

ABBREVIATIONS

Chemotherapy (CT)
Colonic Mucosal Disease index (CMDI)
Cyclooxygenase (COX)
Dihydrofolatereductase(DHFR)
Deoxy ribonucleic acid (DNA)
Glutathione (GSH)
Interleukin (IL)
Lipoxygenase (LOX)
Malonaldehyde (MDA)
Methotrexate (MTX)
Radiotherapy (RT)
Reactive oxygen species (ROS)
Scanning electron microscope (SEM)
Superoxide dismutase (SOD)
Thiobarbituric acid reactive substance (TBARS)
Thromboxane (TX)
Tumour necrosis factor (TNF)

1. INTRODUCTION

Methotrexate (MTX) is an anticancer agent and is a persuasive inhibitor of folic acid. MTX competitively curb dihydrofolate reductase (DHFR) enzyme, who concur in the synthesis of folic acid. Folic acid is requisite for the de novo synthesis of purine [1]. Henceforth, MTX is used as an antimetabolite in cancer chemotherapy including lymphocytic leukemia, non-hodgkin's lymphoma, osteosarcoma, head/neck cancer and mammary gland tumors [2]. In addition MTX is also prescribed for treatment of auxiliary disease conditions like rheumatoid arthritis and refractory inflammatory bowel disease [3]. MTX inhibits the remethylation of homocysteine and this process forms reactive oxygen species, such as superoxide and hydrogen peroxide [4].

MTX associated chemotherapy may induct mucositis directly by reverting Deoxy ribonucleic Acid (DNA) strand break, through fructifying reactive oxygen species (ROS), or through enzymatic or transcription factor incitement in multiple cellular elements within the mucosa. ROS may damage cells, other than cancerous tissues precisely and also enliven secondary mediators of injury, including transcription factors such as nuclear factor-ƙβ (NF-ƙβ) [5]. Activation of transcription factors in response to ROS, radiotherapy, or chemotherapy results in gene up-regulation for tumor necrosis factor-α (TNF-α), interleukins (IL-1β and IL-6) [6]. The antecedent studies has edged towards changes in levels of NF-ƙβ and other proinflammaory cytokines including TNF-α, IL-1β and IL-6 in MTX treated experimental animals as well [7,8]. This leads to tissue injury and apoptosis of cells within the submucosa and primary injury of cells within the basal epithelium and mucositis [9]. In short, the MTX induced injury in the intestinal tissue is conjoined with hyperpolarization, injury to mucosal, submucosal and basal epithelial due to ROS engenderment and consequential up-regulation of pro-inflammatory cytokines/mediators [6].

The MTX is often used as first line disease modifying antirheumatic agent. MTX imparts its effects in rheumatic disorders through modifying the immune system and is commonly prescribed with other drugs, like NSAID's including aspirin [10]. There have been safety concerns in past for using painkillers/ NSAID's/ aspirin along with MTX in patients with inflammatory disorders [11]. The simultaneous use of these drugs with MTX may increase risk of toxicities resulting in issues like mouth ulcers, nausea, vomiting, hepatotoxicity, cardiotoxicity and bone marrow depression [12, 13]. In fact close and periodic monitoring of the various vital parameters is advised in such cases. In view of therapeutic advantage offered by the MTX and

NSAID's/aspirin combination and the risk of associated toxicities, it was considered worth to evaluate the effect of low and high dose of aspirin against MTX administration.

2. MATERIALS AND METHODS

2.1 Animals

Wistar strain of albino rats (both sex) (120-140gm) were procured from central animal house. Animals were kept under controlled environmental condition in polypropylene cage at room temperature (25±2°C) with 12 h light/dark cycle. Animals were fed with standard laboratory animal feed and water *ad libitum*. Animals were conformed to experimental conditions for one week. The experimental protocol was ratified by Institutional Animal Ethics Committee (IAEC) (approval no. UIP/IAEC/2014/FEB/09).

2.2 Drugs and chemicals

MTX (Folitrax-15, IPCA Laboratories Limited Mumbai, India) was purchased from standard commercial supplier and aspirin was purchased from Hi-media, Mumbai, India. The colorimetric kits for serum glutamic oxaloacetic transaminase (SGOT) (catalogue no: 5046622-B-760), serum glutamic pyruvic transaminase (SGPT) (catalogue no: 504622-040-B-809) and (catalogue no: LDH-1159) were procured from Recombigen Laboratories Private Limited, New Delhi and Crest Bio Systems, Goa respectively. The commercial ELISA kits for cyclooxygenase (COX-1 and 2) (catalogue no 760111) and lipoxygenase (15- LOX) (catalogue no 760700) were procured from Cayman Chemicals, Elisaworth, Ann Arbor, MI, USA. All other chemicals were used of analytical grade purchased from Hi-media, Mumbai, India.

2.3 Experimental design

Animals were indiscriminately selected and divided into six groups of six animals each and subjected to treatment as enumerated in Table-1. The MTX (2.5mg/kg, i.p) was administered for initial one week with concomitant administration of aspirin (8 mg/kg, p.o and 45 mg/kg, p.o) for thirteen weeks. Aspirin was preferred to be given by oral route as it is the most common route of administration in humans, thereby providing more translational value to the study. The intraperitoneal route was selected for MTX administration, owing to the fact that it is standardized route to establish intestinal toxicity. After the respective treatment for thirteen weeks, the blood was collected from retro orbital plexus. The blood sample was incubated for 1 h (37°C) and subsequently centrifuged at 10,000 rpm to collect serum. Eventually, animals were sacrificed under light ether anesthesia; the intestinal tissues were collected by securing the both

ends with the help of surgical suture (to avoid drainage of intestinal content). The serum and intestinal tissue, so collected were utilized for further investigations. The high and low doses of the aspirin were selected on the basis of previous literature citing the anti-inflammatory and antiplatelet dose of aspirin to be used in albino rats. [14, 15] Aspirin was solubilised by dissolving in warm water.

2.4 Enzymatic markers for cardiac and liver

The serum samples were scrutinized for the liver (SGOT and SGPT) and cardiac (LDH) marker enzymes using commercial colorimetric assay kits following the manufacturer's protocol.

2.5 Estimations of pH, free acidity and total acidity

The intestinal content was collected and appraised for intestinal pH (Hanna Instruments, HI 98107) free acidity and total acidity, adopting the procedure as described previously [16,17].

2.6 Assessment of colonic mucosal disease index (CMDI)

The colon tissue of approximately 10 cm to anus was taken, opened longitudinally and washed in normal saline buffer and fixed on wax block. The scoring was done and evaluated according to the scores as follow. 0 = normal mucosa, 1= mild hyperemia, no erosion or ulcers on the mucosa surface, 2 = moderate hyperemia, erosion or ulcers appears on the mucosa surface, 3 = severe hyperemia, necrosis and ulcers on the mucosa surface with the ulcerative area less than 40%, 4 = severe hyperemia, necrosis and ulcers on the mucosa surface with the ulcerative area more than 40% [18].

2.7 Biochemical estimations

Intestinal tissues were evaluated for the biochemical parameters for TBARS [19], SOD [20], GSH [21], catalase [22] and protein carbonyl [23] using the established methods at our laboratory.

2.8 Inflammatory cascade enzymes

The intestinal tissues were further evaluated for the enzymatic activities of COX-1, COX-2 and 15-LOX using commercial Elisa kits following the protocol described by the manufacturer using microplate reader [Alere Microplate Reader (AM 2100), Alere Pricate Limited, New Delhi, India].

2.9 Morphological Evaluation

Intestinal tissues from all the groups were appraised for their morphological changes using scanning electron microscopy. Samples were fixed in 2.5% glutaraldehyde for 6h at 4˚C and washed in 0.1M phosphate buffer, for 3 changes each of 15 min at 4˚C. 1% osmium tetroxide

was used as a post fixation for 2h at 4˚C and samples were washed in 0.1M phosphate buffer for 3 changes each of 15 min at 4˚C to remove the unreactive fixative. Specimens were dehydrated by using increasing concentration of acetone viz. 30%, 50%, 70%, 90%, 95%, 100% (dry acetone) to remove water at 4˚C for 30 min period. After that, specimens were air dried (critical point i.e. 31.5 at 1100 psi). The specimens were mounted on to the aluminium stub with adhesive tape. The tissues were contemplated for morphological changes in scanning electron microscope (JEOL-JSM-6490LV) [16].

2.10 Statistical Analysis

All data were presented as mean± SD and analyzed by one way ANOVA followed by Bonferroni test for the possible significance identification between the various groups. *$P<0.05$, **$P<0.01$, ***$P<0.001$ were considered statistically significant. Statistical analysis was carried out using Graph Pad Prism software (3.2), San Diego, CA.

3. RESULTS

The investigation in process manifested a momentous protection by the low dose of aspirin against MTX toxicity. Low dose of aspirin flaunted sententious reduction in the CMDI and restoration of pH, total acidity and free acidity in exemplification to toxic control and high dose of aspirin (Table-1). When appraised biochemically, MTX administration replenished a conspicuous increase in tissue MDA level and the same was reestablished towards normal by aspirin. Furthermore, tissue GSH levels were incomparably increased in MTX treated group (42.95±6.93) in contrast to sham control (16.47±4.47).The enzymatic activity of SOD were contemplated to be marked up after the MTX and aspirin treatment. Furthermore, the enzymatic activity of catalase was drifted down after the aspirin treatment in resemblance to toxic control. When appraised in terms of protein oxidation through protein carbonyl assay the enforcement of antiplatelet dose of aspirin subsidized to move down the protein oxidation as conspicuous through sententious subsidence in protein carbonyl concentration (104.47±1.35) in analogous to toxic control (116.81±0.39) (Table-2). It is conspicuous that biochemically low dose of aspirin exhibited better protection in collation to high dose of aspirin.

The intestinal tissues were figured out for the presence of pro-inflammatory (IL-2) and anti-inflammatory cytokines (IL-4 and IL-10) and the same were observed to be increased after MTX treatment. Concomitant administration of aspirin manifested supplementary rise of the same (Table-3).

Considering the associated cardiac and hepatotoxicity with MTX treatment, the cardiac and liver enzymatic markers were also assayed biochemically. The MTX administration validated compelling toxicity in the cardiac tissue as conspicuously evident by increased MDA generation (0.93±0.01) along with hoisted enzymatic activity of the LDH (323.01±17.17), catalase (22.02±3.16), and SOD (56.77±2.92) corresponding to sham control. Concomitant administration of low dose aspirin marked out a minimal symbolic reassurance of MDA generation and LDH activity. Non-significant reduction in the enzymatic activity of catalase and SOD were contemplated after the aspirin treatment (Table-4).

When perceived through the hepatic enzymes, the MTX was found to be hepatotoxic , with marked up levels of SGOT (44.50±1.95) and SGPT (43.96±0.88) in comparison to control. Treatment with low dose of aspirin accompanied to reinstate the increased level of SGOT whereas successive higher dose was perceived to further augment the enzymatic levels. Besides, aspirin in low as well as high dose further increased the SGPT levels (Table-5). The scanning electron microscopy of the intestinal tissue in control group manifested clear impression of overall villous pattern and mucous (Figur-1) and treatment with MTX depicted hyperpolarization, loss of mucus and distorted villous (Figure-1).

4. DISCUSSION

MTX is one of the extensively used chemotherapeutic agents belonging to antimetabolite category, frequently bracketed with the side effects like intestinal toxicity [24]. In the recent past years, the use of MTX has been protracted from exclusively as a chemotherapeutic agent to antirheumatic and antipsoriatic agent. The extended therapeutic profile of MTX is associated with toxicities including cardiotoxicity, nephrotoxicity and hepatotoxicity [4, 25, 26]. However, one of the uppermost and extensively deliberated toxic effects of MTX is the intestinal damage causing malabsorption and diarrhea, which further results in the severe weight loss and by that disturb the therapeutic regimen[27]. Temperamental mechanisms including inflammation (through generation of pro-inflammatory cytokines), transmutation in the antioxidant defense mechanism (through ROS generation) has been prospected to be susceptive for MTX induced toxicities [6]. Henceforth, the present work was embarked upon to delve into the effect of aspirin (anti-inflammatory agent) against MTX toxicities. Taking into account of the gastrointestinal side effects concurred with the anti-inflammatory (high) dose of aspirin. It was further aforethought to evaluate the antiplatelet (low) dose as well.

Treatment with low dose of aspirin evidenced protection against the intestinal toxicity when observed through physiological parameters. The aspirin treatment proclaimed significant rise in intestinal pH, with decrease in the total and free acidity. The CMDI score in the toxic control was also reduced significantly by aspirin in a dose dependent manner. Prima facie the aspirin was observed to have marked protection against the MTX induced intestinal toxicity.

Diversification in the antioxidant defense mechanisms in the intestinal tissue manipulated to MTX treatment is extensively reported and accepted mechanism for MTX toxicity. A coincidental pattern of results were contemplated in our study as illustrated by increased MDA production in MTX treated group.

The MDA is a product of phospholipid peroxidation and is a marker for oxidative stress. The regimen with aspirin subsidized the MDA production to compelling levels. It would be opportune to mention that the antiplatelet dose was perceived to be more adequate in comparison to anti-inflammatory dose.

The GSH play a paramount role in insulating the tissues from per oxidative attack and MTX incomparably increased the level of tissue GSH which is in contravention to the foregoing findings, suggesting depleted tissues GSH during oxidative stress [28]. The increased GSH level as observed in our study could possibly be credited to increased biogenesis of GSH due to oxidative stress (consequence of feedback mechanism). The concomitant administration with aspirin with MTX demonstrated significant decrease in tissue GSH levels and the same could be imputed either to the restored biogenesis of GSH or diminished levels of oxidative stress as a repercussion to aspirin therapy [29, 30].

The SOD and catalase together aggregate a major team of stockade defense against ROS. SOD forms hydrogen peroxide through its scavenging action on superoxide radical. The superoxide scavenging activity of SOD is further prompted by the catalase (heme protein), which catalyzes the dismutation of hydrogen peroxide to H_2O and molecular oxygen and, by that protects the tissue from highly reactive OH^- radicals. We observed increased enzymatic activity of SOD and catalase which is not in corroborations to the previous studies, as most of the studies have headed for decreased level of SOD and subsequently catalase, as a repercussion of oxidative stress [31]. Notwithstanding, we contemplated increased activity of the SOD and subsequently catalase. One can forecast the diminished activity of SOD and catalase with amelioration of oxidative stress,

however we command the concurrence of physiological compensatory mechanisms to brush off oxidative stress could be one of the possible reasons for such observations.

A simultaneous increase in catalase activity in essential/ requisite with SOD to outsight the superoxide scavenging instituted by SOD, and the same was marked in our study. The ROS can damage all type of biological molecules in addition to lipids, DNA, and proteins. The protein alteration call forth by the direct effect of oxidative stress on amino acid residues can link to the disposition of carbonyl derivatives which is an extensively used marker for protein oxidation [32]. Studying the formation of protein carbonyl have an advantage over lipid peroxidation product, as oxidized proteins are more substantial and therefore the protein carbonyl is universally used as marker of oxidative stress [23]. In the present experiment the MTX accomplished a convincing increase in the protein carbonyl content delineating protein oxidation. The aspirin at antiplatelet dose manifested momentous decrease in the protein carbonyl content in comparison to MTX treated groups. Concomitant administration of aspirin reestablished the levels of physiological antioxidant defense towards normal. The antiplatelet dose of aspirin responded more competently towards the MTX toxicity, in resemblance to high dose.

To have a further perspicacity, we further scrutinized the role of pro and anti-inflammatory cytokines in intestinal tissues and perceived significant increase in the IL-2, IL-4 and IL-10 after the MTX administration.

The IL-2 is a signaling molecule in immune system and oversees the activities of leukocytes and erstwhile lymphocytes that are accountable for immunity. Increased level of IL-2 expression was revealed after the MTX treatment and aspirin (low dose), in addition increased the level of IL-2 when administered collaterally with MTX. On the contrary the high dose of aspirin, accomplished a non- significant decrease in the IL-2 levels with self evident reason of being an anti-inflammatory dose. The present finding is not in affirmation with the previous studies demonstrating diminished IL-2 levels in the serum of the patients/ rheumatoid patients, treated with MTX [33].

A previous study proclaimed that ex-vivo treatment of peripheral blood monocytes with MTX increases expression of IL-4 and IL-10 which is in line with our study. However, aspirin did not demonstrate any curtailment in IL-4 levels neither at high nor at low dose. IL-10 is an anti-inflammatory cytokine and was marked with momentous upsurge after the MTX treatment, advocating the immunocompromised status due to its immunoregulatory properties. The high

dose of aspirin significantly abridged the levels of IL-10, whereas the low dose was acknowledged with heightened IL-10 levels. In augmentation to the other overwhelming toxicities, the MTX has been noted both clinically and preclinically to be cardiotoxic and hepatotoxic [34]. Taking into account of the same and to have a better wisdom, we appraised a serum SGOT and SGPT levels. The results conjectured a momentous hepatotoxicity, as evident through momentous increase in serum SGOT and SGPT levels. The treatment with low dose of aspirin conveyed a compelling protection by normalizing the serum SGOT and SGPT levels. Similarly cardiac toxicity was conspicuous through marked up enzymatic activity of LDH, SOD and catalase along with noticeable embodiment of MDA products after MTX treatment. Aspirin at low dose granted a cogent protection against MTX induced cardiotoxicity. The same could be accredited to the blockade of COX enzyme through inevitable acetylation of serine-529 residue. The COX-1 inhibition in the platelets leads to inhibition of TXA_2 production which is a key platelet aggregator. Moreover in endothelial cells, COX-1 expedite genesis of prostacyclin, which is a vital vasodilator. The dynamic balance of TXA_2 and prostacyclin is modulated by aspirin provides the acclamatory balance [35].

When observed microscopically the MTX administration evidenced significant hyperproliferation, loss of mucus and distorted villous, which is in concordance with the previous reports [16, 36]. The low dose of aspirin manifested significant protection in comparison to MTX and/or aspirin (high dose), either alone or in combination. The microscopic findings in the present investigation suggest that the low dose aspirin can offer advantage over high dose of aspirin to combat intestinal toxicity induced by MTX.

From above one can derive that the low dose of aspirin can contribute a significant preservation against MTX induced intestinal, cardiac, and hepatotoxicity. The said effects of low dose aspirin could be imputed to its antiplatelet action leading to impaired fructification of thromboxane, which binds with platelets to conceive a patch over damaged blood vessels. The patch can eventually be too large to block blood flow, both locally and downstream which is being implicated for the clinical use of aspirin these days [37]. On the contrary the high dose can farther push forward the toxicity. In fact low dose of aspirin can be considered/ evaluated as an adjuvant therapy to MTX treatment regimens. The adjuvant therapy of aspirin with MTX could offer advantages as the renal clearance of MTX or its metabolite 7- hydroxyl MTX is not clinically overwhelmed by aspirin [38, 39]. Henceforth, the adjuvant therapy of low dose of aspirin with MTX

could offer a better pharmacological profile with slackened toxicity. However, use of aspirin in unification with MTX for clinical management of RA is in question and needs to be investigated to its full.

From the above line of evidences, we would like to consummate that the low dose of aspirin can be deliberated as an adjuvant therapy with MTX chemotherapeutic and other treatment regimens, which is expected to provide a surpassing adequacy with attenuated toxicity. However, the competence of MTX and aspirin in clinical management of RA is in question. Notwithstanding, further studies are obligatory to inculcate the expediency of aspirin as an adjuvant to MTX in clinical scenario.

CONFLICT OF INTEREST

No Conflicts declared

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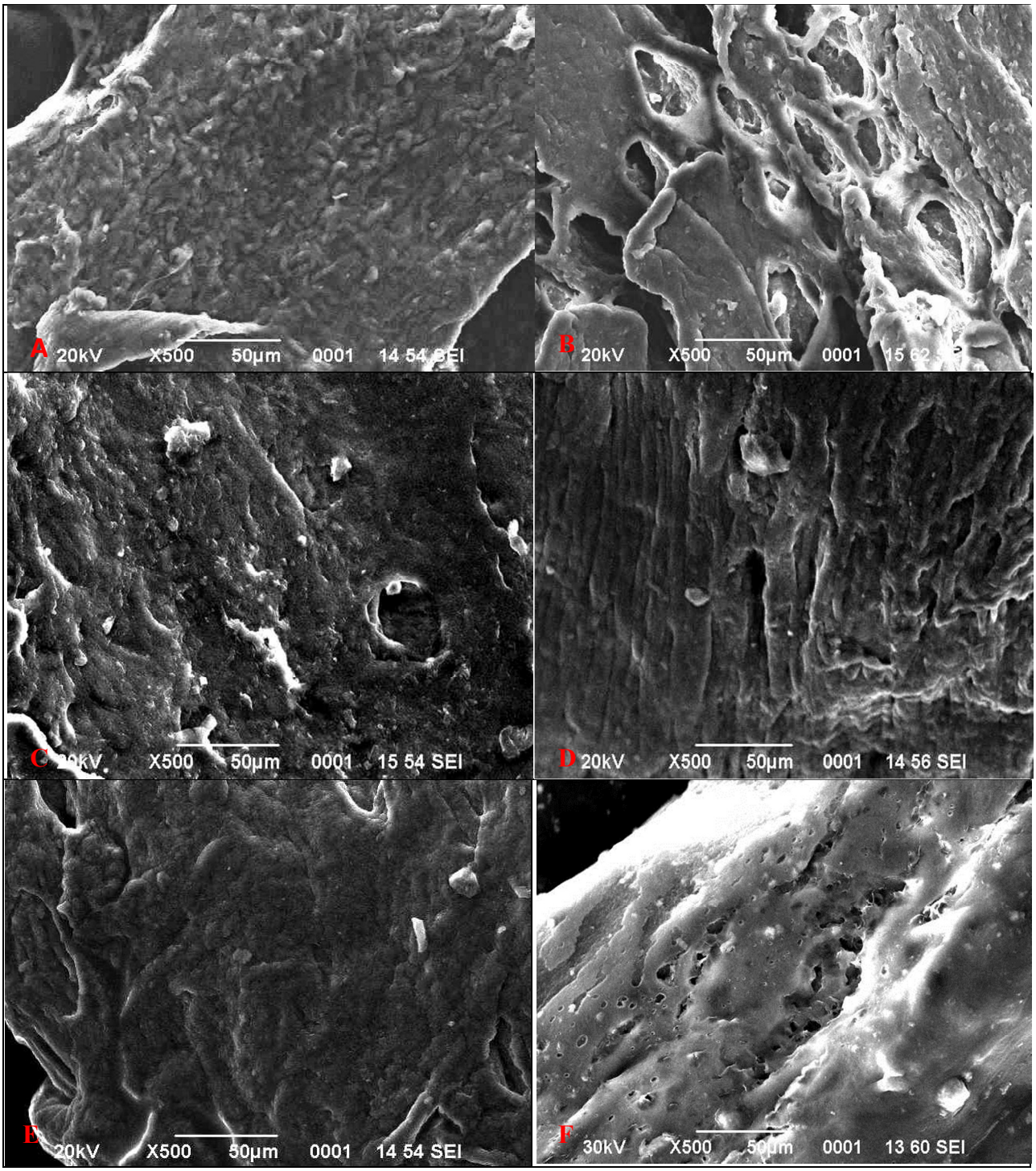

Figure 1: Photomicrographs of the scanning electron microscopy analysis in the treatment groups.

A) Control group (3mi/kg p.o, normal saline)
B) Toxic control(MTX,2.5mg.kg ,i.p)
C) Aspirin (8mg/kg, p.o).
D) Aspirin (8mg/kg,po)+MTX(2.5mg/kg,ip)
E) Aspirin (45mg/kg, p.o)
F) Aspirin (45mg/kg, p.o) + MTX(2.5 mg/kg,i.p)

Table:-1 Effect of aspirin on pH, total acidity, free acidity and CMDI in MTX induced intestinal damage

S. No.	Group	Treatment	**pH**	**Total Acidity**(mEq/l)	**Free Acidity**(mEq/l)	**CMDI**
1.	Group-I	Sham control(Normal saline,3.0ml/kg, po)	7.15±0.02	10.99±1.67	7.32±1.96	0.17±0.40
2.	Group-II	Toxic control (MTX,2.5ml/kg,ip)	6.27±0.04	14.38±1.42	11.92±0.92	4.00±0.00
3.	Group-III	Aspirin (8mg/kg, po)	7.67±0.02***	10.52±0.45*** (26.84%)	7.94±0.45*** (33.38%)	0.83±0.40*** (79.25%)
4.	Group-IV	Aspirin+ MTX (8mg/kg,po+2.5ml/kg, ip)	7.40±0.02***	10.52±0.43*** (26.84%)	7.70±0.51*** (35.40%)	1.83±0.40*** (54.25%)
5.	Group-V	Aspirin (45mg/kg, po)	7.73±0.06***	11.37±0.64*** (20.93%)	8.88±0.58*** (25.50%)	1.83±0.40*** (54.25%)
6.	Group-VI	Aspirin+ MTX (45mg/kg,po+2.5ml/kg,ip)	7.50±0.02***	14.47±0.77 (0.76%)	11.40±0.77 (4.36%)	2.83±0.75** (29.25%)

Each group contains six animals. Values are represented as mean ± SD.
Statistical significance compared to toxic control using one-way ANOVA followed by Bonferroni test (*$p<0.05$, **$p<0.01$, and ***$p<0.001$).
Values in parenthesis represent percentage inhibition

Table-2: Effect of aspirin on TBARS, GSH, SOD, Catalase and Protein carbonyl in MTX induced intestinal toxicity.

S.No.	Group	TBARS (nm of MDA/ mg ofprotein)	GSH (mg %,1×10^{-4})	SOD (unit of SOD/mg of protein)	Catalase (nm at H_2O_2/min/mg of protein)	Protein carbonyl (Nanomoles/ml)
1	Group-I	0.71±0.04	22.40±0.55	16.47±4.47	7.70±0.81	50.45±0.39
2	Group-II	2.91±0.02	113.32±0.01	42.95±6.93	37.46±1.60	116.81±0.39
3	Group-III	0.79±0.01**	26.7±2.44**	26.73±6.89**	8.57±1.01***	70.40±1.55***
4	Group-IV	1.35±0.01***	61.8±3.13***	48.74±8.14***	23.07±2.27***	104.47±1.35***
5	Group-V	1.25±0.01***	28.2±2.82***	37.21±7.23***	31.11±2.91***	94.76±3.92***
6	Group-VI	1.47±0.02***	98.7±4.79***	44.52±9.50***	34.62±7.30***	109.50±2.00*

Each group contains six animals. Values are represented as mean ± SD.
Statistical significance compared to toxic control using one-way ANOVA followed by Bonferroni test.
*$p<0.05$, **$p<0.01$, and ***$p<0.001$ were considered statistically significant.

Table-3: Effect of aspirin on proinflammatory and anti-inflammatory cytokines in MTX induced intestinal toxicity.

S.no.	Group	IL-2(pg/ml)	IL-4(pg/ml)	IL-10(pg/ml)
1	Group-I	524.80±0.00	108.05±5.19	82.66±5.95
2	Group-II	764.82±115.94	238.86±46.84	876.98±213.07
3	Group-III	526.99±156.71	257.31±41.94	177.82±0.00***
4	Group-IV	968.17±133.23**	300.08±49.92**	1167.60±79.*
5	Group-V	483.01±31.46*	284.28±15.34	1699.10±66.96**
6	Group-VI	683.66±70.41	223.87±0.00	224.91±25.92**

Each group contains six animals. Values are represented as mean ± SD.
Statistical significance compared to toxic control using one-way ANOVA followed by Bonferroni test : compare all pairs of columns,*$p<0.05$, **$p<0.01$, ***$p<0.001$ were considered statistically significant.

Table-4: Effect of aspirin on LDH, TBARS, Catalase and SOD in MTX induced toxicity.

S.NO.	Groups	LDH (unit/litre)	TBARS (nm of MDA/ mg of protein)	Catalase (nm at H_2O_2/min/mg of protein)	SOD (unit of SOD/mg of protein)
1	Group-I	226.66±11.44	0.48±0.03	5.27±0.36	19.16±8.67
2	Group-II	311.66±17.17	0.93±0.01	22.02±3.16	56.77±2.92
3	Group-III	239.80±4.24*	0.55±0.01**	4.84±0.51**	25.92±0.41***
4	Group-IV	307.21±0.57**	0.79±0.02***	19.46±2.41	53.98±2.21
5	Group-V	266.85±4.97	0.62±0.01**	7.16±4.02***	33.93±12.11***
6	Group-VI	403.30±9.67**	0.86±0.03**	29.75±10.07	56.87±2.76

Each group contains six animals. Values are represented as mean ± SD.
Statistical significance compared to toxic control using one-way ANOVA followed by Bonferroni test
*$p<0.05$, **$p<0.01$, ***$p<0.001$ were considered statistically significant.

Table-5: Effect of aspirin on liver marker enzymes in MTX induced toxicity.

S. No.	Group	**SGOT** (unit/dl)	**SGPT** (unit/dl)
1	Group-I	23.34±0.20	20.88±1.51
2	Group-II	44.50±1.95	43.96±0.88
3	Group-III	24.69±1.01***	30.49±0.64**
4	Group-IV	30.38±1.25**	52.65±0.98**
5	Group-V	40.62±0.66**	36.21±3.77**
6	Group-VI	58.70±1.80***	65.73±1.79***

Each group contains six animals. Values are represented as mean ± SD.
Statistical significance compared to toxic control using one-way ANOVA followed by Bonferroni test: compare all pairs of columns,*$p<0.05$, **$p<0.01$, and ***$p<0.001$ were considered statistically significant.