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Research Paper

Review: Mechanisms of methotrexate induced small intestinal injury and its prevention- an update

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I. MTX uses

Methotrexate (MTX), a structural analogue of folic acid, is one of the most widely used therapeutic agents available to treat many solid tumors, hematologic malignancies, and autoimmune diseases [1,2]. It is currently the most common anti-rheumatic drugs prescribed for the treatment of rheumatoid arthritis and other rheumatic disorders [3]. MTX earned a new indication with its efficacy in the treatment for refractory inflammatory bowel disease [4]. Patients suffering from psoriasis have benefited from MTX in addition to those with atopic dermatitis, chronic urticarial infection [5].

Methotrexate is administered at doses that range from 12 mg intrathecally and 20 mg/m^2 orally, intramuscularly, or intravenously as weekly maintenance chemotherapy for acute lymphocytic leukemia (ALL) to doses as high as 33,000 mg/m^2 intravenously for some other indications [6]. Doses of 500 mg/m^2 or higher given intravenously are defined as high-dose methotrexate (HDMTX) and are used to treat a variety of adult and pediatric cancers, including ALL, osteosarcoma, and lymphomas [7-9].

II. Gastrointestinal side effects of MTX

In addition to cancer cells being affected by MTX, rapid proliferating cells such as bone marrow and gastrointestinal cells are also affected. Adverse reactions from long-term low-dose MTX treatment may be present in 30% to 80% of patients, and acute toxicity in patients taking MTX at low doses may, in some cases, be life-threatening [10]. HDMTX therapy can cause significant toxicity, which not only leads to morbidity and occasional mortality but may also interrupt cancer treatment, potentially leading to inferior anticancer outcomes. Its cytotoxic structure causes life-threatening side effects, such as intestinal injury, and as a result of this, use of this agent is often limited. Gastrointestinal toxicity is one of the most frequently observed side effects that may lead to a reduction in the dose or even a discontinuation of the drug [11,12].

Chemotherapy induced mucositis is a debilitating, dose-limiting, and costly side effect of cancer therapy. Mucositis occurs in 40% of cancer patients after standard doses of treatment and in almost 100% of patients treated with high doses of chemotherapy [13] and can affect the entire gastrointestinal tract causing discomfort, nausea, vomiting, bloating, diarrhoea, ulceration, bleeding and in some cases result in septicaemia [14].It, leads to dose reduction or prevention of continuation of selected therapies, prolongs hospital stay, increases re-admission rates, increases healthcare cost, compromises patients' nutritional status, impairs patients' quality of life, and is occasionally fatal .Generally, methotrexate-induced gastrointestinal mucositis is a moderately severe but common side effect in routine clinical use [15].The small intestinal damage (enteritis) induced by MTX treatment results in malabsorption and diarrhea [16,17]]. Approximately 60% of cancer patients that receive a chemotherapy treatment that includes MTX experience diarrhea and malabsorption [18]. This malabsorption results in weight loss and disturbs the cancer chemotherapy of patients.

III. Mechanisms of methotrexate induced intestinal injury

The exact mechanism of intestinal toxicity caused by MTX is not fully understood. However, it is reported that MTX could cause intestinal damage via producing reactive oxygen species (ROS) , reactive nitrogen species (RNS) and activation of NF-κB [19,20]. NF-κB regulates the production of numerous cytokines and mediates cell damage, which can be activated by ROS generation [21,22]. The production of inflammatory cytokines such as TNF- α,IL-1β, and IL-6 is provoked by ROS production [23]. Also, MTX administration leads to inflammatory cascades involving the activation of NF-κB, with increased expression of pro-inflammatory cytokines such as IL-6 and TNF-α, followed by activation of the JAK/ STAT3signaling [24,25]. Upon JAK/STAT phosphorylation, it translocates to the nucleus, binds with the target gene promoter region, and provokes the transcription of genes involved in the inflammatory reactions [26]

3.1. Role of oxido-nitrosative stress and mitochondrial damage in MTX induced small intestinal injury

Several studies have shown that reactive oxygen species , reactive nitrogen species, nitro-oxidative stress [27-36], mitochondrial damage[37-39] and epithelial cell apoptosis play important roles in MTX induced small intestinal injury in animal models [40-46]. When intestinal damage is triggered by MTX, increased ROS production and depletion of antioxidant defense mechanism plays a crucial role [47].ROS mediates lipid peroxidation, which leads to tissue damage development after MTX administration. This degradation of cell membranes impairs normal cellular activities [48]. The antioxidant glutathione (GSH) content in cells was lowered and cytosolic peroxide was elevated following MTX treatment [49]. Multiple previous studies showed that MTX treatment altered redox status in the small intestine and increased intestinal ROS biomarkers such as malondialdehyde [50-52]. The direct toxic effects of MTX are thought to be caused by excessive generation of free radicals, causing an imbalance between free radical production and antioxidant defense, which finally results in the development of oxidative stress [48,53].

Oxidative stress causes necroptosis and apoptosis in enterocytes, as well as the destruction of the intestinal structure [54,55]. In addition, cytoskeletal proteins and other cellular proteins are damaged by excess of free radicals in the intestinal epithelium. Furthermore, it increases intestinal permeability, which makes it more likely for microorganisms and antigens from the luminal environment to enter the bloodstream and increase the risk of systemic reaction syndrome [56]

We have been working on the elucidation of the mechanism of MTX induced intestinal toxicity, using rat model, over the past 13 years. We have demonstrated that increased nitro-oxidative stress , peroxynitrite overproduction and protein tyrosine nitration, ,enterocyte apoptosis and necrosis, mitochondrial damage, upregulation of mitochondrial apoptotic pathway .We have shown that methotrexate administration induces differential and selective protein tyrosine nitration and cysteine nitrosylation in the subcellular organelles of the small intestinal mucosa of rats.

These are our significant findings

1. Methotrexate administration causes increased apoptosis and necrosis of epithelial cells and marked neutrophil infiltration as evidenced by four fold increase in myeloperoxidase (MPO) activity in the small intestines of rats. Activation of the mitochondrial apoptotic pathway contributes to methotrexate-induced small intestinal injury in rats [57]

2. Alteration in antioxidant defense mechanisms in the small intestines of methotrexate treated rat may contribute to its gastrointestinal toxicity

Tissue reduced glutathione, protein thiol and the activities of glutathione reductase, superoxide dismutase and catalase were significantly decreased in the intestines of MTX treated rats as compared with controls. Nitrotyrosine, measured immunohistochemically was detected in all the parts of the small intestine. On the other hand the activities of glutathione peroxidase, glutathione S transferase, protein carbonyl content,malondialdehyde, and conjugated dienes were significantly increased in the intestines of MTX treated rats. The results of the study suggest that alteration in antioxidant enzymes may contribute to enhanced oxidative stress in the intestines of MTX treated rats and hence small intestinal damage. [58]

3. Increased nitrosative stress may play a critical role in MT induced small intestinal injury

Inducible nitric oxde synthase expression was increased in the small intestines of MTX treated rats. Nitrotyrosine, the foot print of peroxynitrite production measured immunohistochemically was detected in all the parts of the small intestine. Duodenum stained the most for nitrotyrosine, followed by ileum and then jejunum. These findings reveal that iNOS induction and peroxynitrite overproduction -nitrosative stress may play a critical role in methotrexate induced small intestinal damage [59]

4. Methotrexate administration induces differential and selective protein tyrosine nitration and cysteine nitrosylation in the subcellular organelles of the small intestinal mucosa of rats
Tyrosine nitrated proteins and cysteine nitrosylated proteins were determined in the subcellular organelles fractions of mucosa using immunoprecipitation and western blot. The proteins in the subcellular fractions were separated by 1D electrophoresis, and probed with anti -nitrotyrosine antibody and anti-nitrosocysteine antibody. After MTX treatment, a general increase in protein tyrosine nitration as well as a change in the spectrum of proteins that underwent nitration was observed. The relative densities of the 3 nitrotyrosine protein adducts were as follows: Mitochondria > cytosol > microsomes > nucleus. In the mitochondrial fraction increased nitration of 12 kDa, 25 kDa 29Kda, 47 kDa, and 62Kda proteins, in the cytosol increased nitration of 12 kDa, 19 kDa, 45 kDa, and 60 kDa proteins and in the nuclear fraction increased nitration of 17 kDa, 35 kDa, and 58 kDa proteins was observed. On the other hand, MTX treatment resulted to a general decrease in protein cysteine nitrosylation in all the subcellular fractions. These results suggest that MTX induced, PON mediated small intestinal injury is mediated by differential nitration and nitrosylation of proteins in the subcellular organelles with increased protein tyrosine nitration and decreased cysteine nitrosylation[60]

6. Mitochondrial dysfunction and respiratory chain defects in a rodent model of methotrexate-induced enteritis
Respiratory control ratio, the single most useful and reliable test of mitochondrial function, and 3-(4,5-dimethylthiazol-2-yll)-2,5-diphenyltetrazolium bromide reduction, a measure of cell viability were significantly reduced in all the fractions of MTX-treated rat enterocytes. A massive decrease (nearly 70%) in the activities of complexes II and IV was also observed. The results of the present study suggest that MTX-induced damage to enterocyte mitochondria may play a critical role in enteritis [61].

7. Melatonin pretreatment protected against methotrexate-induced small intestinal damage in rats. The protective effect is mediated by attenuation of oxido- nitrosative stress, protein tyrosine nitration, and PARP activation [62].

3.2.Nuclear factor erythroid‑2‑related factor 2(Nrf2)‑Kelch‑like ECH‑associated protein 1 (Keap1) pathway and intestinal injury by MTX
The primary regulator of cellular responses to external stressors is nuclear factor erythroid-2-related factor 2 (Nrf2) [63]. The nuclear factor erythroid-2-related factor 2 gene is responsible for encoding antioxidants and detoxification enzymes providing a redox sensing system [64]. Previous studies have reported the involvement of Nrf2 in MTX-induced intestinal injury [65].

3.3 Role of inflammation in MTX‑induced intestinal injury

Three main alterations take place during acute inflammation.1. increased capillary permeability, which permits larger serum molecules to enter the tissues.2. increased leukocyte migration into the tissue and 3.increased blood flow to the affected area [66].NF-kB is a key regulator of inflammation that is involved in the synthesis of inflammatory mediators and activation of pro-inflammatory cytokines [67]. NF-κB regulates the expression of numerous immune system components and modulates inflammation [68]. Among these are pro-inflammatory cytokines, chemokines, and inducible enzymes such as nitric oxide synthase (iNOS) and cycloxygenase-2 (COX-2). Moreover, NF-κB regulates cytokines including IL-2and IL-12 that affect the proliferation and differentiation of lymphocytes. It is also currently believed that pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6, may also play a role in MTX-induced intestinal damage [69,70].

We have demonstrated that PARP overactivation and activation of NF-κB-iNOS-COX2-TNF α inflammatory signaling pathway play important role in MTX induced small intestinal injury. MTX treatment resulted in NFκB activation and nuclear translocation as evidenced by immunofluorescence, immunohistochemistry , and western blot. NFκB mRNA espression was also increased. There was increased protein and mRNA expressions of NFκB target genes, TNF-α, iNOS, COX-2, PLA2, HO-1, HSP70, MMPs 2 and Aminoguanidine pretreatment attenuated MTX induced activation of NFκB and its proinflammatory target genes and improved MTX induced morphological changes[61b,62b] .

3.4.Role of JAK/STAT3/SOCS3 in MTX‑induced small intestinal injury

JAK/STAT is the signalling pathway for many cytokines and growth factor production . When cytokines such as IL-6 bind to JAK/STAT3, STAT3 becomes phosphorylated. After nuclear translocation, the phosphorylated form of STAT3 acts as a transcriptional factor that upregulates the genes related to inflammation) [71,72]. Studies have shown that MTX administration provoked JAK1 and STAT3 phosphorylationin rat models of intestinal injury.

The regulation of the JAK/STAT system involves numerous mechanisms, one of which is the control of JAK kinase activity phosphorylation by suppressor of cytokine signaling (SOCS) proteins [73]. The most

important member of the SOCS family is SOCS3, which can block JAK/STAT3 signaling inresponse to mitosis, growth factors, and cytokines . SOCS3 has ability to reduce JAK phosphorylation by inhibiting JAK kinase binding and competing with JAK to prevent STAT3 phosphorylation [74] .Previous study showed that MTX administration resulted decline in SOCS3 level [75] .

3.5.Involvement of peroxisome proliferator-activatedreceptor-gamma (PPAR-γ) in MTX-induced intestinal toxicity

It is widely known that PPAR-γ is a powerful inhibitor of ROS and inflammation. Its conformation changes upon activation preventing the production of pro-inflammatory mediators, which in turn prevents a range of inflammatory responses [76]. The reduction of NF-kB, STAT1, and AP-1 transcriptional activity is one of the PPAR-γ mediated anti-inflammatory actions [77 .The decreased level of PPAR-γ is associated with increased oxidative stress in a rat model of duodenal injury induced by MTX [78,79].

Although several attempts have been made to investigate the mechanism of methotrexate induced gastrointestinal toxicity, the underlying biological events contributing to the pathogenesis of mucositis are still being defined and multiple pathways leading to epithelial cell death seem likely.

IV. Therapeutic protection against methotrexate-induced intestinal injury

Several natural and synthetic compounds have been tried to attenuate MTX induced small intestinal mucositis as discussed below

Aminoguanidine

Aminoguaidine (AG) is an inducible nitric oxide synthase (iNOS) inhibitor, which can regulate the activity and expression levels of iNOS [80]. Leitao et al have demonstrated that inhibition of iNOS by aminguanidine prevents MTX induced small intestinal injury in rats [81] .We have demonstrated that pretreatment with aminoguanidine had a dose-dependent protective effect on MTX-induced mucositis. AG pretreatment reduced iNOS protein levels, mucosal nitric oxide levels, and protein tyrosine nitration. AG pretreatment also restored the activities of electron transport chain (ETC) complexes, vital tricarboxylic acid (TCA cycle) enzymes, and mitochondrial antioxidant enzymes [61b].

Melatonin

More commonly known as the sleep hormone, melatonin also has antioxidant, anti-inflammatory, antiapoptotic, and many other crucial properties [82,83]. Melatonin, a pineal hormone functions as a high-capacity antioxidant, or free radical scavenger, within mitochondria, playing a dual role in combating cellular oxidative stress. Firstly, it directly neutralizes free radicals, and secondly, it promotes the gene expression of essential antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase. The direct antioxidant and free radical scavenging properties of melatonin are mainly due to its electron-rich aromatic indole ring, which makes it a potent electron donor that can significantly reduce oxidative stress [83,84]. We have shown that melatonin protects against methotrexate-induced small intestinal damage. Its effect is mediated by attenuation of oxido-nitrosative stress, protein tyrosine nitration, and PARP activation (62,85a,b).

Natural compounds

Many natural compounds have been tried to ameliorate MTX mucositis in animal models.

Rutin

Rutin is one of the main flavonoid glycosides present in fruits and fruit peels, mainly in citrus fruits such as lemons and oranges [86]. It possesses several pharmacological activities, including the ability to effectively scavenge superoxide radicals and act as an immunomodulator, anti-inflammatory, antioxidant, and anti-carcinogenic [87,88] . The mechanism of action of rutin is due to its to its antioxidant capacity through the Nrf2/ARE and anti-inflammatory properties due to NF-κB, COX-2, IL-6, and TNF-α suppression. Studies have shown that rutin is able to attenuate intestinal oxidative stress changes by lowering intestinal MDA and elevating GSH content and restoring SOD activity. Rutin attenuated MTX-induced intestinal inflammation, as proved by decreased IL-2 and increased IL-4 and IL-10. In addition, rutin was found to inhibit the enzymatic activity of COX and lipoxygenase. Thus rutin, has protective effect against MTX-induced intestinal injury [89].

Hesperidin

Hesperidin is a flavanone group member, [90]. Citrus fruits, including lemon, orange, and grapefruit, are a good source of this natural antioxidant compound [91,92]. It exhibited antiapoptotic, anti-inflammatory, and anti-carcinogenic properties together with its antioxidant activity [93-95]. Pretreatment with hesperidin before

MTX administration attenuated intestinal injuries by decreasing oxidative stress and myeloperoxidase activity. It also inhibited iNOS activity and reduced IL8 levels [96].

Lycopene

Tomatoes and other red fruits have a high concentration of the red pigment lycopene. Lycopene has the ability to scavenge ROS[97,98]and has anti-inflammatory . properties [99].Lycopene protects against MTX induced intestinal injury by decreasing the levels of IL-1β, and oxidative stress [100].

Quercetin

Of all the flavonoids, quercetin is the most widely distributed. Apples, potatoes, soybeans, and other fruits and vegetables are rich sources of quercetin [101]. Quercetin is a powerful antioxidant that protects against ROS and has been shown to reduce MTX induced mucosal injury in rats [102].

Apocynin

Apocynin (APO) is a natural organic methoxy-substituted catechol compound that acts as an antioxidant. [103]. APO reduced intestinal oxidative stress by decreasing intestinal MDA and increasing SOD activity and GSH content in MTX treated rats. APO exhibited powerful anti-inflammatory by inhibiting the production of NF-κB mRNA and decreasing pro-inflammatory cytokine levels together with upregulating anti-inflammatory PPAR-γ proteins [104].

Taurine

Numerous studies have proved that taurine has antioxidant and antiinflammatory properties. Previous studies have demonstrated the protective effect of taurine against MTX-induced intestinal injury through a variety of mechanisms including modulation of Keap1/Nrf2/HO-1 signals , inhibition of the NF-κB/iNOS signal and reduction of caspase 3 expression [105].

Omega‑3 polyunsaturated fatty acids

Omega-3 fatty acids have anti-inflammatory and antioxidant properties [106,107]. Omega-3 polyunsaturated fatty acids (PUFAs) exhibited potential protective effect against MTX-induced apoptosis in intestinal mucosa [108]. Rats treated with MTX and omega-3 PUFA- showed a significant decrease in enterocyte apoptosis together with reduced numbers of macrophages in conjunction with lower levels of COX-2, TNF-α, and NF -κB in the mucosa of treated rats [108].

Umbelliferone

A naturally occurring member of the coumarin family, umbelliferone (UMB) or 7-hydroxycoumarin, is present in a wide variety of plants, including garden angelica, coriander, and carrots [109]. Numerous investigations have determined that UMB possesses biological properties, including anti-inflammatory [110], antioxidant[111,112] and anticancer effects [113]. Studies have shown that UMB protects against MTX induced intestinal damage by inhibiting oxidative stress, as shown by decrease in MDA contents and the elevation of Nrf2, SOD, HO-1, and GSH levels. Additionally, it inhibited STAT3, NF-κB, IL-6, and TNF-α levels, thereby exhibiting anti -inflammatory effects.

Other compounds

Compounds including salecan, carnitine, β glucan, N acetylcysteine, β carotene, proanthocyanidine, ozone, ginger, lycopene, turmeric, resveratrol, arginine, and vitamin E have been shown to protect against MTX induced small intestinal injury in animals[114-118].

V. Conclusions

Despite its longstanding recognition, frequency, and clinical impact, treatment options for MTX induced intestinal mucositis are not satisfactory. Many natural and synthetic compounds have been tried to ameliorate MTX mucositis in animal models. But none of them offer complete, mechanism based protection. Therefore, there are no FDA approved drugs for the treatment of intestinal mucositis. The management of intestinal mucositis largely involves the control of symptoms using antibiotics, anaesthetics, and analgesics. Thus, there is an urgent need to develop drugs that minimise GI toxicity.

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