Potential Therapeutic Activities of Melatonin and Alpha Lipoic Acid on Methotrexate-Induced Hepatotoxicity in Rats

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Abstract. The search for newer and better hepatoprotective agents is imperative so as to alleviate the socio-economic burden of hepatotoxicity caused by MTX. This study investigated the protective effects of melatonin (MT) and alpha lipoic acid (ALA) against MTX-induced hepatotoxicity in albino rats. Forty-eight adult male albino rats randomized into four groups were used. The rats in group C were treated intraperitoneally (ip) with MT (10mg/kg), ALA (10mg/kg) and MT+ALA daily for 5 days. The rats in group D were pretreated ip with MT, ALA and MT+ALA daily for 5 days prior to treatment with a dose of MTX (20mg/kg) ip on the 5th day. The rats in group A (control) were treated ip with 0.3mL of normal saline daily for 5 days whereas rats in group B were treated ip with a dose of MTX (20mg/kg) on the 5th day. On day 6, rats were anesthetized; serum samples were extracted from blood samples and assessed for liver function parameters. Liver samples were assessed for biochemical parameters and histology. Hepatic distortions in MTX-treated rats were characterized by significant (p<0.05) increases in serum aminotransferases, gamma glutamyl transferase, alkaline phosphatase, lactate dehydrogenase, bilirubin, and liver malondialdehyde levels with significant (p<0.05) decreases in liver superoxide dismutase, catalase, glutathione and glutathione peroxidase levels when compared to control. Liver histology showed hepatocyte necrosis in MTX-treated rat. Interestingly, MTX-induced hepatotoxicity was significantly attenuated in MT (p<0.05) and ALA (p<0.05) pretreated rats when compared to MTX-treated rats. However, pretreatment with MT+ALA produced most significant (p<0.05) attenuation when compared to MT and ALA respectively. MT and ALA may have clinical benefits in hepatotoxicity caused by MTX.

Keywords: Methotrexate, Liver Toxicity, Antioxidant, Protection, Rat

1. Introduction

Methotrexate (MTX) is one of the chemotherapeutic agents used for the treatment of cancer. It is also a well-established and effective treatment for rheumatic, psoriasis and bowel diseases (Crohn’s disease). Despite the success achieved with its use, its hepatotoxic effect has attracted considerable attention. The frequency by which MTX causes hepatotoxicity varies however, an incidence of 7–11 per 100 patients have been reported [1]. MTX undergoes hepatic and intracellular metabolism to polyglutamated forms which can accumulate in the liver causing hepatotoxicity [2]. Common forms of hepatic injury associated with MTX include mild hepatitis, cholestasis, fatty liver, fibrosis, cirrhosis and acute liver failure. Also, alteration in serum liver biochemistry is one of the earliest signs of hepatotoxicity caused by MTX [3]. Furthermore, studies using animal models have reported impairments in liver redox status characterized by decreases in superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) activities. Also, up-regulations in the hepatic activities of lipid peroxidation (LPO) indexes have been reported [4].
Melatonin (N-acetyl-5-methoxytryptamine) (MT) is produced by the pineal gland, and plays an important function by regulating neuroimmuno-endocrine system [5]. It is involved in many essential physiological functions such as the regulation of inflammation, circadian rhythms as well as functioning as an antioxidant with a broad spectrum [6]. Its antioxidant effect includes scavenging and neutralization of oxidative and nitrosative radicals and up-regulation of the functions of enzymes involved in the inhibition of oxidative and nitrosative stress. It has the ability to extensively inhibit oxidative processes both in lipid and aqueous environments. Numerous studies have reported its propensity to inhibit oxidative damage to biomolecules such as lipids, proteins and DNA [7]. Furthermore, it has shown to be effective in animal models of diseases such as arthritis, psychosis, cancer, diabetes and obesity [8] and has shown potential hepatoprotective activity against different animal models of liver injury [9].

Alpha lipoic acid (thioctic acid) (ALA) is a natural occurring chemical substance that has a five-member cyclic disulphide and hydrocarbon tail ending with a carboxylic acid group [10]. It occurs as a prosthetic group in alpha-keto acid dehydrogenase complexes of mitochondria which plays an important function in energy production and metabolism [11]. When administered, it is taken-up by cells where it is reduced to dihydrolipoic acid (DHLA) and exerts its antioxidant effect both intracellularly and extracellularly. It is an amphipathic molecule which antioxidant effect occurs in hydrophilic and lipophilic environments [12]. It also has the ability to inhibit lipid peroxidation (LPO) and facilitate the functions of some antioxidants [13]. Furthermore, it has shown potential therapeutic effects in diseases such as hypertension, asthma, cancer, and hyperlipidemia induced in animals [14]. Additionally, it has abrogated liver damage associated with metal intoxication, alcohol, carbon tetrachloride and mushroom poisoning in animal models [15]. In view of the aforementioned information, this study evaluated the protective activities of MT and ALA against MTX-induced hepatotoxicity in albino rats.

2. Materials and Methods

2.1. Drug/chemicals and animals. MT and ALA were supplied by AO Pharm Import and Export Co Ltd, China whereas MTX was manufactured by Biochem Pharmaceutical Industries Ltd, India. Forty-eight adult male albino rats (200–220 g) were sourced from the animal breeding house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, Niger Delta University, Nigeria. The rats were kept in cages of 6 rats per cage under standard conditions (12 Light: 12 Day) at a temperature of 25–26°C and were fed *ad libitum* with water and commercial rodent diet. The rats were allowed to acclimatize to laboratory condition for two weeks prior to the study. This study was approved by the Research Ethics Committee of the Department of Pharmacology/Toxicology, Faculty of Pharmacy, Niger Delta University, Nigeria. The rats were handled according to the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science.

2.2. Animal treatment

- The albino rats were randomized into four (4) groups A-D.
- Group A (Control) contained 6 rats which were treated intraperitoneally (ip) with 0.3mL of normal saline daily for 5 days
- Group B contained 18 rats which were divided into sub-groups (B1- B3) of six rats each and were treated with MT (10mg/kg), ALA (10mg/kg) [16] and MT+ALA ip daily respectively for 5 days
- Group C contained 6 rats which were treated with a dose of MTX (20mg/kg) [17] ip on the 5th day
- Group D contained 18 rats which were divided into sub-groups (D1- D3) of six rats each and were pretreated ip with MT (10mg/kg), ALA (10mg/kg) and MT+ALA daily for 5 days before treatment with a dose of MTX (20mg/kg) ip on the 5th day respectively.

2.3. Animal sacrifice and evaluation of parameters. The rats were anesthetized with diethyl ether; blood samples were collected from the heart and allowed to clot. The clots were centrifuged (1500g, for 15 min) and serum samples were extracted for biochemical analyses. Liver samples were excised and quickly rinsed in ice-cold saline and homogenized in 0.1 M Tris-HCl buffer, pH 7.4 and centrifuged at 1200g for 15 min. The supernatants were decanted and used for the evaluation of biochemical parameters. Serum and liver levels of alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), total bilirubin (TB), and conjugated bilirubin (CB) were evaluated using commercial test kits (Randox Laboratories UK). Liver malondialdehyde (MDA) was measured according to Buege and Aust, 1978 [18]. Superoxide dismutase (SOD) was measured as described by Sedlak and Lindsay, 1968 [19] whereas catalase (CAT) was assayed according to the method of Aebi, 1984 [20]. Glutathione peroxidase (GPx) was assayed as reported by Rotruck et al. 1973 [21] whereas total protein was analyzed as described by Lowry et al. 1951 [22].

2.3.1. Histological analysis. Liver samples were excised after rats were sacrificed. Liver samples were rinsed and fixed in 10% formal saline. The fixed liver samples were processed and embedded in paraffin blocks. The liver tissues were sectioned (5 µm), prepared and stained with Hematoxylin and Eosin (H&E). Stained sections were examined for pathology with the aid of a light microscope.
2.4. Statistical analysis. Values are expressed as mean± standard error of mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey’s multiple comparison test. Significance was set at p<0.05

Table 1: Activities of melatonin and alpha lipoic acid on liver function markers of methotrexate-treated rats.

| Treatment          | ALT (U/L) | AST (U/L) | ALP (U/L) | LDH (U/L) | GGT (U/L) | TB (g/dL) | CB (g/dL) |
|--------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Control            | 25.6±2.00abde | 22.4±2.22bcd | 27.6±3.33bcde | 23.5±3.00bcd | 0.21±0.01bde | 4.66±0.11bcde | 2.00±0.22bde |
| MT                 | 24.1±2.90abcd | 22.1±2.53bcd | 26.9±2.29bcde | 23.1±2.51bcd | 0.20±0.06bde | 4.52±0.32bcde | 2.10±0.06bcde |
| ALA                | 24.5±2.47bcde | 21.3±2.19bcd | 26.5±2.43bcde | 22.9±2.22bcd | 0.21±0.01bde | 4.56±0.14bcde | 2.14±0.09bcde |
| MT + ALA           | 25.0±2.01bcd | 21.0±2.97bcd | 25.9±2.56bcde | 22.4±2.01bcd | 0.20±0.09bde | 4.44±0.32bcde | 2.17±0.07bcde |
| MTX                | 88.9±6.44acde | 93.7±5.43acde | 99.5±6.11acde | 110.1±7.11acde | 0.90±0.05acde | 13.6±0.21acde | 8.72±1.00acde |
| MT + MTX           | 58.0±2.21abcd | 62.2±4.00bcd | 69.1±4.33abcd | 73.9±6.62abcd | 0.60±0.07abcd | 9.72±0.32abcd | 6.70±0.52abcd |
| MTX + ALA + MTX    | 40.1±3.51abcde | 42.0±3.62abcde | 49.5±3.41abcde | 46.4±3.13abcde | 0.45±0.09abcde | 6.23±0.50abcde | 4.61±0.44abcde |

MTX: Methotrexate, MT: Melatonin, ALA: Alpha Lipoic Acid, n=6, Data are expressed as Mean± SEM. aDifference at p<0.05 when compared to control, bDifference at p<0.05 when compared MTX, cDifference at p<0.05 when compared MT+MTX, dDifference at p<0.05 when compared ALA+MTX, eDifference at p<0.05 when compared MT+ALA+MTX

Table 2: Activities of melatonin and alpha lipoic acid on liver tissue biochemical indexes of methotrexate-treated rats.

| Treatment          | ALT (U/L) | AST (U/L) | ALP (U/L) | GGT (U/L) | LDH (U/L) |
|--------------------|-----------|-----------|-----------|-----------|-----------|
| Control            | 200.5±12.8bcd | 255.6±10.1bcd | 190.1±8.43bcd | 160.0±9.54bcd | 30.8±2.00bcd |
| MT                 | 197.0±11.6bcd | 245.5±12.3bcd | 185.6±9.00bcd | 152.2±8.11bcd | 29.0±2.15bcd |
| ALA                | 195.7±11.7bcd | 250.3±10.7bcd | 189.4±8.01bcd | 150.6±9.50bcd | 30.0±2.43bcd |
| MTX                | 190.8±10.8bcd | 235.0±11.6bcd | 179.7±9.54bcd | 147.4±10.2bcd | 29.5±2.00bcd |
| MTX                | 785.3±15.4acde | 893.6±14.0acde | 699.3±15.1acde | 690.5±18.1acde | 95.9±6.14acde |
| MTX + ALX          | 401.7±12.3abce | 525.2±10.2abde | 373.1±10.4abde | 339.3±11.8abde | 65.3±3.62abde |
| ALA + MTX          | 417.5±10.7abde | 421.5±11.5abcde | 380.4±11.6abde | 350.1±12.1abde | 50.1±4.41abcde |
| MTX + ALX + MTX    | 215.4±10.5bcd | 320.6±12.6bcd | 200.0±10.2bcd | 170.9±10.5bcd | 37.0±2.32bcd |

MTX: Methotrexate, MT: Melatonin, ALA: Alpha Lipoic Acid, n=6, Data are expressed as Mean± SEM. aDifference at p<0.05 when compared to control, bDifference at p<0.05 when compared MTX, cDifference at p<0.05 when compared MT+MTX, dDifference at p<0.05 when compared ALA+MTX, eDifference at p<0.05 when compared MT+ALA+MTX

Table 3: Activities of melatonin, and alpha lipoic acid on liver oxidative stress indexes of methotrexate-treated rats.

| Treatment          | MDA (nmole/mgprotein) | CAT (U/mgprotein) | SOD (U/mgprotein) | GSH (μmole/mgprotein) | GPx (U/mgprotein) |
|--------------------|-----------------------|-------------------|-----------------|-----------------------|------------------|
| Control            | 0.36±0.01bcd          | 41.6±2.55bde      | 25.4±2.12bcde   | 10.4±0.33bcd          | 20.8±1.67bcde    |
| MT                 | 0.34±0.07bcd          | 42.1±3.12bcde     | 26.1±1.21bcd    | 10.8±0.42bcd          | 22.5±2.63bcd     |
| ALA                | 0.35±0.09bcd          | 42.6±4.41bcde     | 25.5±2.54bcde   | 10.6±0.15bcd          | 21.6±2.44bcde    |
| MTX                | 0.34±0.04bcd          | 43.5±3.00bcde     | 28.2±2.00bcd    | 12.0±0.42bcd          | 22.0±2.45bcd     |
| MTX                | 1.97±0.03acde         | 12.7±0.45acde     | 7.25±0.42acde   | 2.42±0.11acde         | 6.38±0.40acde    |
| MTX + ALX          | 0.98±0.08abde         | 17.1±1.27abde     | 15.1±1.65abde   | 5.49±0.15abde         | 9.01±1.33abde    |
| ALA + MTX          | 0.60±0.02abcde        | 23.6±1.33abcde    | 13.4±1.11abcde  | 5.32±0.66abcde        | 11.4±0.15abcde   |
| MTX + ALA + MTX    | 0.37±0.05bcd          | 32.8±2.19abcd     | 24.2±1.43abcd   | 9.93±0.37abcd         | 16.5±1.63abcd    |

MTX: Methotrexate, MT: Melatonin, ALA: Alpha Lipoic Acid, n=6, Data are expressed as Mean± SEM. aDifference at p<0.05 when compared to control, bDifference at p<0.05 when compared MTX, cDifference at p<0.05 when compared MT+MTX, dDifference at p<0.05 when compared ALA+MTX, eDifference at p<0.05 when compared MT+ALA+MTX
3. Results

3.1. Effect on serum liver function indexes. Serum AST, ALT, ALP, GGT, LDH, TB and CB levels were normal (P>0.05) in rats treated with MT and ALA when compared to control (Table 1). However, serum AST, ALT, ALP, GGT, LDH, TB and CB levels were significantly (p<0.05) increased in rats treated with MTX when compared to control (Table 1). The increases in the aforementioned parameters represent 247.3%, 318.3%, 260.5%, 368.5%, 328.6%, 225.0% and 336.0% respectively. In contrast, the levels of the aforementioned parameters were significantly (p<0.05) decreased in rats pretreated with individual doses of MT and ALA respectively when compared to MTX-treated rats. However, serum AST, ALT, ALP, GGT, LDH, TB and CB levels were significantly (p<0.05) decreased in rats pretreated with MT+ALA when compared to rats pretreated with individual doses of MT and ALA respectively (Table 1).

3.2. Effect on liver tissue biochemical parameters. Normal (P>0.05) liver AST, ALT, ALP, GGT, and LDH levels were obtained in rats treated with MT and ALA when compared to control (Table 2). In contrast, significant (p<0.05) increases in liver AST, ALT, ALP, GGT, and LDH levels were obtained in rats treated with MTX in comparison to control. The observed increases in liver AST, ALT, ALP, GGT, and LDH levels represent 291.7%, 249.6%, 267.9%, 331.6%, and 311.4% respectively (Table 2). However, liver AST, ALT, ALP, GGT, and LDH levels were significantly (p<0.05) decreased in rats supplemented with individual doses of MT and ALA respectively when compared to MTX-treated rats. The decreases in the aforementioned parameters were most significant (p<0.05) in rats pretreated with MT+ALA when compared to rats pretreated with individual doses of MT and ALA respectively (Table 2).

3.3. Effect on liver oxidative stress markers. The effects on liver SOD, CAT, GSH, GPx and MDA levels were not significant (P>0.05) in rats treated with MT and ALA when compared to control (Table 3). On the other hand, liver SOD, CAT, GSH and GPx levels were significantly (p<0.05) decreased whereas MDA levels were significantly (p<0.05) increased in rats treated with MTX when compared to control (Table 3). It is of interest to know that liver SOD, CAT, GSH, GPx levels were significantly increased (p<0.05) whereas MDA levels were significantly (p<0.05) decreased in rats pretreated with individual doses of MT and ALA respectively when compared to MTX- treated rats. Furthermore effects on the aforementioned parameters were most significant (p<0.05) in rats pretreated with MT+ALA when compared to pretreatment with individual doses of MT and ALA respectively (Table 3).

3.4. Effect on liver histology. Normal liver histology was observed in control rat (Figure 1A) whereas hepatocyte necrosis was observed in rat treated with MTX (Figure 1B). Also, hepatocyte necroses were observed in rats pretreated with MT (Figure 1C) and ALA (Figure 1D) respectively. However, normal liver histology was observed in rat pretreated with MT+ ALA (Figure 1E).

4. Discussion

The search for newer and better hepatoprotective agents is imperative so as to alleviate the socio-economic burden associated with hepatotoxicity caused by MTX. This study investigated the protective effects of melatonin (MT) and alpha lipoic acid (ALA) against MTX-induced hepatotoxicity in rats. Clinically, biochemical cut-offs for serum ALT, AST, GGT, LDH CT, and TB are yardsticks for hepatic dysfunction [23]. Normal levels of serum and liver AST, ALP, ALT, GGT, LDH CT, and TB were obtained in rats treated with MT and ALA. On the other hand, the serum and liver levels of the aforementioned indexes were elevated in MTX-treated rats. The observation in MTX treated rats is in agreement with earlier findings [24]. However, decreases in AST, ALP, ALT, GGT, LDH CT, and TB were noted in rats supplemented with individual doses of MT and ALA. Interestingly, supplementation with MT+ALA produced the best reductions in the levels of the aforementioned parameters. The observation in MTX-treated rats can be attributed to altered hepatic membrane porosity or hepatic cell death which might have lead to perturbations in carrier mediated transport, activities of membrane bound enzymes, receptor binding, endocytosis and exocytosis, stimulating the discharge of hepatic biochemical parameters in to the blood [25].

The antioxidant defense system is a free radical scavenging system that consists of antioxidants such as superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH). It is an essential and primary protective mechanism responsible for the regulation of the excess activities of free radicals in cells by scavenging and neutralizing them to prevent oxidative and nitrosative stress associated damage. Therefore, oxidative/nitrosative stress can occur as a consequence of increased free radical production or reduced antioxidant defense [26]. Normal liver antioxidant defense system was observed in rats treated with MT and ALA as shown by normal levels of SOD, CAT, GSH and GPx. In contrast, hepatic antioxidant defense was compromised in rats treated with MTX due to abysmal low levels of SOD, CAT, GSH and GPx. The observation in rats treated with MTX is consistent with previous findings [27]. However, hepatic antioxidant defense was up-regulated in rats supplemented with MT and ALA which was characterized by increased SOD, CAT, GSH and GPx levels.

The evaluation of MDA level is used as an essential indicator of LPO in various diseases and xenobiotic-induced toxicities. MDA produced during enzymatic and
oxygen radical-induced LPO can react with DNA to form MDA-DNA adducts causing damage to DNA and other biomolecules. Also, since lipids present in cells are responsible for maintaining the integrity of cellular membranes, extensive LPO can alter composition and denature lipid membranes and cause outflow of cellular contents [28]. In this study, hepatic MDA levels were normal in rats treated with MT and ALA. In contrast, up-regulations in...
MDA levels were observed in rats treated with MTX. The observation in MTX-treated rats is a clear sign of hepatic LPO which is consistent with previous reports [29]. However, hepatic MDA levels were decreased in rats supplemented with individual doses of MT and ALA with most decrease obtained in rats supplemented with MT+ALA. Studies have reported fatty infiltration, inflammation, necrosis, and varying degrees of fibrosis in hepatotoxicity caused by MTX [30]. This study observed hepatocyte necrosis in rat treated with MTX. Interestingly, hepatocyte necrosis was completely abrogated in rats pretreated with MT+ALA. The observed hepatoprotective effects of MT and ALA in this study can be correlated with the reported protective effects of MT and ALA against lopinavir/ritonavir-induced hepatotoxicity in albino rats [31]. MTX-induced hepatotoxicity has been attributed to the precipitation of MTX and/or 7-hydroxy MTX in the liver. Also, previous studies have explained the role of free radicals in the pathogenesis of MTX-induced hepatotoxicity. MTX can generate free radicals which can damage hepatic biomolecules such as proteins, lipids and DNA [32]. MTX can incapacitate hydrofolic reductase enzyme, thereby preventing the transformation of folic acid to folinic acid leading to the inhibition of the synthesis of some amino and nucleic acids. This may cause damage to organelles and plasma membranes of hepatic parenchyma cells interfering with their functions and allowing leakage of enzymes [33]. The protective effects of MT and ALA observed in this study can be attributed to their antioxidant activities by scavenging and neutralizing free radicals and stimulating other antioxidant activities in the liver of MTX-treated rats. MT can localize itself in a superficial position within the lipid bi-layers of membrane phospholipids where it can effectively scavenge free radicals, preventing LPO and thus provide an indirect means by which membranes can resist oxidative damage. Also, MT can stabilize cell membrane fluidity thereby preserving their functional efficiency [34]. Furthermore, MT and ALA might have protected the liver by increasing bile production and improving bile/bilirubin excretion through improvement in hepatic ATP activity, thereby facilitating the functions of ATP dependent transporters require for bile flow and secretion [35]. The most hepatoprotective activity observed in MT+ALA pretreated rats can be attributed to complimentary antioxidant effects.

### 5. Conclusion

This study suggests that MT and ALA may be used to treat or prevent hepatotoxicity caused by MTX.

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### Competing Interests

The authors declare no competing interests.

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