World Journal of

Gastrointestinal Pharmacology and Therapeutics

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Contents

ABOUT COVER

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AIMS AND SCOPE

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WJGPT mainly publishes articles reporting research results obtained in the field of gastrointestinal pharmacology and therapeutics and covering a wide range of topics including acid-related disorders, functional gastrointestinal disorders, fundamentals of gastrointestinal pharmacology, *etc.*

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Basic Study

Melatonin restores zinc levels, activates the Keap1/Nrf2 pathway, and modulates endoplasmic reticular stress and HSP in rats with chronic hepatotoxicity

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Abstract

BACKGROUND
Melatonin (MLT) is a potent antioxidant molecule that is shown to have a beneficial effect in various pathological situations, due to its action against free radicals.

AIM
To evaluate the effect of MLT on carbon tetrachloride (CCl_4) induced liver injury in rats in terms of oxidative stress, reticular stress, and cell damage.

METHODS
Twenty male Wistar rats (230-250 g) were divided into four groups: Control rats, rats treated with MLT alone, rats treated with CCl_4 alone, and rats treated with CCl_4 plus MLT. CCl_4 was administered as follows: Ten doses every 5 d, ten every 4 d, and seven every 3 d. MLT was administered intraperitoneally at a dose of 20 mg/kg from the 10th wk to the end of the experiment (16th wk).

RESULTS
MLT was able to reduce the release of liver enzymes in the bloodstream and to decrease oxidative stress in CCl$_4$ treated rats by decreasing the level of thiobarbituric acid reactive substances and increasing superoxide dismutase activity, with a lower reduction in serum zinc levels, guaranteeing a reduction in liver damage; additionally, it increased the expression of nuclear factor (erythroid-derived 2)-like 2 and decreased the expression of Kelch-like ECH-associated protein 1. MLT also decreased the expression of the proteins associated with endoplasmic reticulum stress, *i.e.,* glucose-regulated protein 78 and activating transcription factor 6, as well as of heat shock factor 1 and heat shock protein 70.

CONCLUSION
MLT has a hepatoprotective effect in an experimental model of CCl$_4$-induced liver injury, since it reduces oxidative stress, restores zinc levels, and modulates endoplasmic reticulum stress.

Core Tip: Liver cirrhosis is a chronic condition of the liver that is characterized by inflammation, steatosis, and formation of fibrotic tissue that impair liver function. Several studies have demonstrated the relationship between oxidative stress and the development of different diseases. As oxidative stress can damage lipids, proteins, and DNA, causing changes in cell homeostasis, it is important to study therapeutic substances that can minimize or delay the effects of the disease. Hepatotoxic drugs are commonly used as an experimental model to assess different stages of liver disease. Melatonin was used in this work as a therapeutic strategy and showed hepatoprotective action in a chronic hepatotoxicity model.

INTRODUCTION

Chronic liver diseases are characterized by multistep processes that involve several molecules and cellular events to transform a normal parenchyma into a parenchyma with steatosis, increased collagen deposition, fibrosis, and cirrhosis[1]. Many studies have demonstrated the presence of overproduction of free radicals and reactive oxygen species (ROS) in inflammatory chronic diseases. ROS are able to oxidize macromolecules or activate transcription factors[2-4]. The relation between the development of chronic liver diseases and ROS has been widely discussed since oxidative stress may cause damage in lipid, protein, and DNA, producing alterations in cellular redox homeostasis[5].

Cellular homeostasis can be disrupted by a variety of stimuli, including metabolic imbalance, oxidative stress, and folding of malformed proteins. In response to these stressors, cells induce specific molecular pathways that usually involve the activation of signaling cascades or changes in gene expression. These responses allow cells to adapt to stress and to regain homeostasis. However, if stress is intense or prolonged, the cells are unable to reestablish homeostasis and, in turn, activate pathways that result in cell death[6].

Carbon tetrachloride (CCl$_4$) is a hepatotoxic drug used in experimental models to evaluate different stages of liver disease and thus define therapeutic strategies. Exposure to CCl$_4$ have a toxic effect on liver cells, promoting damage in tissue and causing changes in the antioxidant defense mechanism. This process results in an imbalance between ROS production and antioxidative enzymes release[5].

The antioxidant defense mechanism is dependent on some minerals, including zinc. This mineral is essential for a large number of structural proteins, enzymatic processes, and transcription factors. Its deficiency causes numerous clinical manifestations, such as appetite loss, smell and taste disturbances, cerebral and immune dysfunction, and reduced drug elimination capacity. These clinical characteristics have been observed in chronic liver diseases[7].

The alteration in cellular redox homeostasis either results in mitochondrial dysfunction or can affect other organelles, such as the endoplasmic reticulum (ER). The ER stress may impair protein synthesis, resulting in accumulation of misfolded proteins. ER stress induces the activation of the intracellular

signaling pathway called unfolded protein response (UPR), which contributes to the pathogenesis of several chronic diseases[8,9].

Melatonin (N-acetyl-5-methoxytryptamine; MLT) is an indoleamine lipophilic derivative of tryptophan. It is produced primarily by the pineal gland of vertebrates and is also detected in other organs[10-13]. MLT has effects on sleep, mood, sexual maturation and reproduction, immune function, aging, and the antioxidative defense system[14,15], and exhibits numerous actions, including anti-inflammatory, antioxidant, and oncostatic properties. In hepatocytes, it was observed that MLT protects from oxidative damage. Its action is based on scavenging the free radicals and stimulating antioxidant enzymes[15-17]. Reducing the generation of ROS would be a way to slow the progression of cell damage observed in liver diseases.

Our aim was to study the effects of MLT on biochemical indexes, serum zinc levels, and oxidative stress in rats exposed to CCl_4. Also, we evaluated the expression of proteins involved in cell damage, ER stress, and unfolded protein response in animals with liver injury induced by CCl_4 and treated with MLT.

MATERIALS AND METHODS

Animal experiments and drug treatment

Twenty male Wistar rats with an average weight of 230-250 g were used. The animals were housed at 22 °C with 12 h light-dark cycles, had free access to water, and received a restricted diet (16 g of chow per day for each animal).

All experiments were performed in accordance with the Guiding Principles for Research Involving Animals (NAS) and the Committee of Ethics and Research in Health of the Graduate and Research Group of Hospital de Clinicas de Porto Alegre under protocol number 100316.

The animals were divided into four groups: Control (CO) rats, rats treated with MLT alone (MLT), rats treated with CCl_4 alone (CCl_4), and rats treated with CCl_4 plus MLT (CCl_4 + MLT). The CCl_4 and CCl_4 + MLT groups received 27 intraperitoneal doses of 0.5 mL of CCl_4 dissolved in mineral oil (1:6). The first ten doses were given at an interval of 5 d, the following ten at an interval of 4 d, and the last seven at an interval of 3 d[18]. In order to promote cytochrome P450 enzyme induction, phenobarbital was added to the drinking water of each animal at a concentration of 0.3 g/L 7 d before the first application and throughout the experiment[19].

MLT (Sigma Aldrich, St. Louis, MO) was administered intraperitoneally to the MLT and CCl_4 + MLT groups at a dose of 20 mg/kg/day from the 10th wk to the end of the experiment (16th wk)[20].

At 24 h after the last administration of CCl_4, the animals were anesthetized with 1% xylazine and 10% ketamine, and then we collected blood samples from the retro-orbital plexus. Liver samples were obtained for the remaining analyses. At the end of the experiment, the animals were killed under deep anesthesia by exsanguination, as described in the guidelines of the American Veterinary Medical Association (AVMA) on Euthanasia (AVMA, 2007).

Biochemical analysis

Serum levels of alanine aminotransferase (ALT) (U/L) and aspartate aminotransferase (AST) (U/L) were determined by the kinetic UV test. Alkaline phosphatase (AP) (U/L) was quantified by the colorimetric kinetic test. The levels of these enzymes were measured using routine laboratory methods of the Hospital de Clinicas de Porto Alegre by enzymatic methods (automated - Siemens Advia 1800 Chemistry system).

Lipid peroxidation and cytosolic superoxide dismutase

Frozen tissue from each rat was homogenized in ice-cold phosphate buffer (KCl 140 mmol/L, phosphate 20 mmol/L, pH 7.4) and centrifuged at 3000 rpm for 10 min. Protein concentration in liver homogenates was determined using a bovine albumin solution[21]. Lipid peroxidation (LPO) was determined by measuring the concentration of thiobarbituric acid reactive substances (TBARS) (nmol/mg protein)[22]. Spectrophotometric absorbance in the supernatant was measured at 535 nm. Cytosolic superoxide dismutase (SOD) (EC 1.15.1.1) was assayed as described previously[23]. The auto-oxidation rate of epinephrine, which is progressively inhibited by increasing amounts of SOD in the homogenate, was monitored spectrophotometrically at 560 nm. The amount of enzyme that inhibited 50% of epinephrine auto-oxidation was defined as 1 U of SOD activity.

Western blot analysis

Western blot analysis was performed using cytosolic and nuclear extracts prepared from liver homogenates as previously described[24]. The supernatant fraction was collected and stored at -80 °C in aliquots until use. Protein concentration was measured as described previously[21]. Lysate proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes[25]. The membranes were then blocked with 5%

nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS) for 1 h at room temperature and probed overnight at 4 ºC with polyclonal antibodies against nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (SC30915/57kDa), Kelch-like ECH-associated protein 1 (Keap1) (SC33569/69kDa), activating transcription factor 6 (ATF6) (SC166659/90kDa), and 78-kDa glucose-regulated protein (GRP78/BiP) (SC376768/78kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, United States) at a dilution of 1:200-1000 with TTBS in 5% nonfat dry milk. Antibodies against heat shock factor 1 (HSF1) (H4163/75kDa) and heat shock protein 70 (HSP70) (H5147/73 and 72kDa) (Sigma Aldrich, St Louis, MO, United States) were used at a dilution of 1:5000 with TTBS in 5% nonfat dry milk, as well as antibodies against β-actin (A5060/42kDa) and anti-glyceraldehyde 3-phosphate dehydrogenase (G9545/37kDa) (Sigma Aldrich, St Louis, MO, United States) at a dilution of 1:2,000 with TTBS in 5% nonfat dry milk. After washing with TTBS, the membranes were incubated for 1 h at room temperature with secondary HRP conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States, 1:4000). Protein detection was performed *via* chemiluminescence using a commercial ECL kit (Amersham Pharmacia Biotech, Little Chalfont, UK)[24]. The density of the specific bands was quantified with imaging densitometer software (Scion Image, Maryland, MA).

Zinc measurement
The serum zinc concentration was measured using a Zinc Assay Kit (Abnova Corporation, Taipei City, Taiwan). The absorbance (425 nm) of the supernatant was measured by spectrophotometry, and the values are expressed in μg/dL.

Statistical analysis
The mean and standard deviation (SD) were calculated for all data. Significant differences between means were evaluated by one-way analysis of variance (ANOVA). Statistical significance was assessed using Tukey's test. P values < 0.05 were deemed significant. All analyses were carried out using Statistical Package for Social Sciences (SPSS), version 18.0 (SPSS Inc., Chicago, IL).

RESULTS

Effect of MLT on biochemical analysis
We evaluated the hepatoprotective activity of MLT against CCl_4-induced liver injury in rats (Table 1). Analyses of transaminase enzymes showed that in the CCl_4 group, AST and ALT were increased compared to the CO, MLT, and CCl_4 + MLT groups. Also, AP demonstrated a significant increase in the CCl_4 group compared to the other groups, indicating the presence of hepatocellular injury. Animals treated with MLT showed reduced enzymatic levels when compared to the CCl_4 group ($P < 0.001$).

Effect of MLT on LPO and SOD activity
Determination of lipid peroxidation in liver tissue with liver injury induced by CCl_4 and treated with MLT was performed by the TBARS method, which showed an increase of malondialdehyde (MDA) formation in tissues exposed to CCl_4. Lipid peroxidation was significantly increased in the CCl_4 group (+ 61%) *vs* control animals. The CCl_4 + MLT group had LPO levels similar to the controls (Table 1). SOD activity was lower in the CCl_4 group (- 27%) compared to animals of the control group. However, treatment with MLT reduced LPO and restored SOD activity in the CCl_4 + MLT group in comparison with the CCl_4 group (Table 1).

Effect of MLT on Keap1/Nrf2 pathway regulation
Protein markers related to oxidative stress were also evaluated. Animals in the CCl_4 group overexpressed Keap1 and underexpressed nuclear factor Nrf2. Conversely, animals in the CCl_4 + MLT group underexpressed Keap1 and overexpressed Nrf2 (Figure 1). The overexpression of Keap1 observed in the CCl_4 group suggested an inhibitory action on nuclear factor Nrf2. However, the underexpression of Keap1 evidenced in the CCl_4 + MLT group suggested that MLT shows a cytoprotective effect on hepatocytes by stimulating the action of Nrf2.

Effect of MLT on endoplasmic reticular stress
Concerning markers of ER stress, ATF6 and GRP78/BiP expression was strongly increased in animals treated with CCl_4 ($P < 0.05$), while that in the CCl_4 + MLT group had expression similar to the control group (Figure 2).

### Effect of MLT on HSP70 and HSF1 expression
Stressful insults, such as exposure to toxic agents, stimulate HSF1 to act as a master activator of the response of HSPs, including HSP70[26]. HSF1 and HSP70 expression was significantly higher in animals from the CCl_4 group compared with the control group and significantly reduced in animals from the CCl_4 + MLT group compared with the CCl_4 group (Figure 3).

Table 1 Effect of melatonin on liver function, lipid peroxidation, superoxide dismutase activity, and zinc level

Parameter	CO	MLT	CCl$_4$	CCl$_4$ + MLT
AST (U/L)	175.4 ± 34.4	161.8 ± 20.3	1016.8 ± 340.8[a]	519.6 ± 127.5
ALT (U/L)	50.2 ± 5.6	43.8 ± 6.6	270 ± 90.8[a]	177 ± 42.7
AP (U/L)	80.2 ± 25.4	75 ± 14.3	395 ± 130.8[a]	238 ± 24.5
TBARS (nmol/mg protein)	0.18 ± 0.01	0.15 ± 0.01	0.29 ± 0.03[a]	0.18 ± 0.05
SOD (U/SOD/mg protein)	12.84 ± 1.08	11.43 ± 0.7	9.32 ± 0.3[a]	13.18 ± 1.6
Zinc (μg/dL)	48.66 ± 4.9	0.62 ± 10.4	11.11 ± 4.31[a]	28.96 ± 6.67

[a]$P < 0.001$, carbon tetrachloride (CCl$_4$) group *vs* other groups. Data are expressed as the mean ± SD. CO: Control group; Melatonin (MLT): Rats receiving MLT alone; CCl$_4$: Rats treated with CCl$_4$ alone; CCl$_4$ + MLT: Rats treated with CCl$_4$ plus MLT; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AP: Alkaline phosphatase; TBARS: Thiobarbituric acid reactive substances; SOD: Superoxide dismutase.
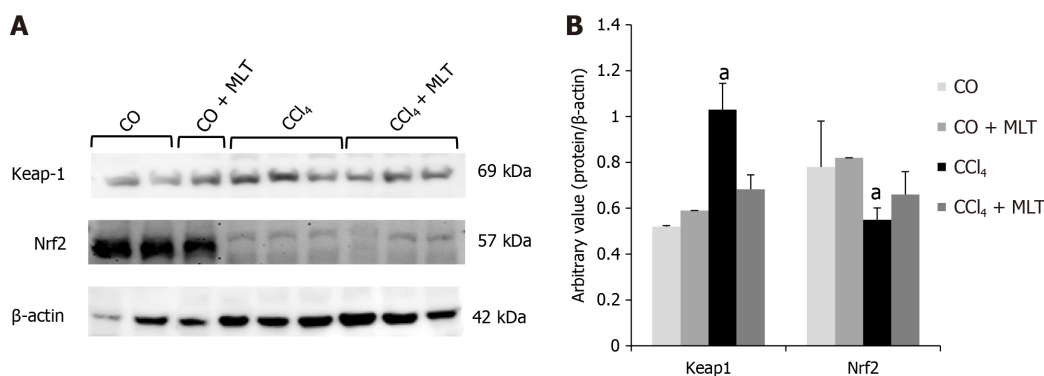
Figure 1 Western blot analysis. A: Cytoplasmic and nuclear fractions were analyzed by Western blot with antibodies against Kelchlike-ECH-associated protein 1, nuclear factor (erythroid-derived 2)-like 2, and β-actin; B: Arbitrary values expressed as the mean and standard deviation. [a]$P < 0.05$ CCl$_4$ *vs* other groups. MLT: Melatonin.
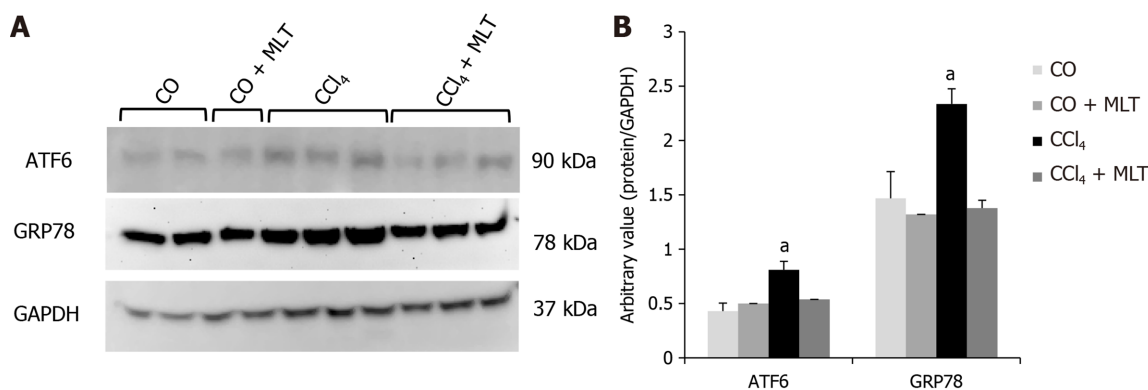
Figure 2 Western blot analysis. A: Nuclear and cytoplasmic fractions were analyzed by Western blot with antibodies against activating transcription factor 6, glucose-regulated protein 78, and glyceraldehyde 3-phosphate dehydrogenase; B: Arbitrary values expressed as the mean and standard deviation. [a]$P < 0.05$ CCl$_4$ *vs* other groups. MLT: Melatonin.

Effect of MLT on serum zinc levels

When assessing serum zinc levels in different groups, we observed that the induction of liver injury by CCl$_4$ caused damage to hepatocytes associated with a significant reduction in zinc levels. In contrast, animals treated with CCl$_4$ + MLT showed zinc levels similar to those in the control group, indicating that MLT has a hepatoprotective effect (Table 1).
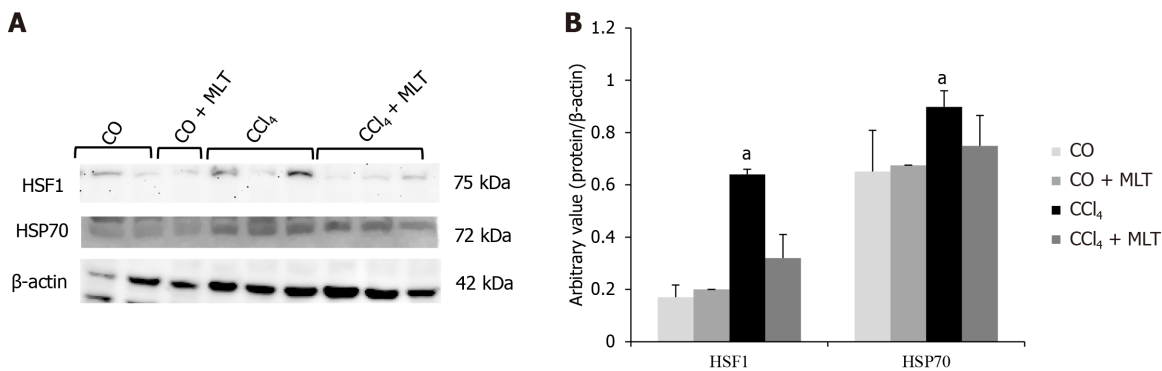
Figure 3 Western blot analysis. A: Nuclear and cytoplasmic fractions were analyzed by Western blot with antibodies against heat shock factor 1, heat-shock protein (HSP70), and β-actin; B: Arbitrary values expressed as the mean and standard deviation. ${}^a P < 0.05$ CCl₄ *vs* other groups. MLT: Melatonin.

DISCUSSION

Many studies have demonstrated the presence of overproduction of free radicals and ROS in chronic liver diseases. ROS are able to oxidize macromolecules or activate transcription factors, resulting in oxidative stress and ER stress[27,28,29] Our study evidenced that MLT treatment in rats with liver injury chemically induced by CCl₄ was promising. We observed that MLT was able to activate the Nrf2 pathway and modulate the unfolded protein response and ER stress in rats with liver injury. MLT plays a key role in hepatoprotection, which results in the reduction of hepatic enzymatic levels and oxidative stress.

We evaluated the efficacy of MLT regarding the protection of macromolecules against the oxidative damage during hepatotoxicity process in animals exposed to CCl₄. In general, MLT consistently improved the pattern of the hepatic enzymes. Also, this study observed a reduction of oxidative stress in animals that were treated with MLT. With respect to oxidative damage, MLT stimulated the Keap1/Nrf2 pathway and restored the normal expression of the proteins involved in the ER stress.

In the present study, chronic exposure to CCl₄ induced liver injury, as evidenced by the significant increase in the liver enzymes under study. MLT treatment protected the liver from toxicity promoted by the toxic agents (CCl₄). AST and ALT in the CCl₄ group increased significantly compared to the other groups. This analysis characterized a condition of necrosis or alteration of cell membrane permeability. This increase is indicative of acute liver injury and hepatocellular damage[30]. Animals treated with CCl₄ + MLT had significantly reduced AST and ALT activities, indicating a significant cytoprotective effect for MLT on hepatocytes[31]. Other studies reported that the use of hepatotoxic agents such as CCl₄ and thioacetamide to generate free radicals affects the permeability of hepatocyte membranes and increases serum levels of enzymatic biochemical parameters. The use of antioxidants such as quercetin was able to reduce the release of these enzymes, probably due to the restoration of liver parenchyma[32,33].

The involvement of ROS in liver injury and in the death of hepatocytes exposed to toxic agents has been extensively documented both *in vitro* and *in vivo*[8]. It is believed that the changes in redox homeostasis may play a significant role in the pathogenesis of many diseases characterized by chronic inflammation, activation of wound healing, and fibrogenesis[27,28,32,34]. The mechanism of CCl₄ toxicity occurs through the generation of highly toxic free radicals. In the present study, this evidence is reinforced by the increase in TBARS levels in the CCl₄ group. Among many effects attributed to MLT, we highlight its potent antioxidant effect[20,35-37]. Our results showed that MLT significantly reduced liver LPO, which is consistent with previous reports indicating that MLT is capable of removing free radicals and has a protective effect in experimentally induced hepatotoxicity[20,37,38]. There is evidence that MLT increases the efficiency of the electron transport chain and, as a consequence, reduces the generation of ROS[39,40]. This antioxidant property is due to the double bond existing in its chemical structure, which allows electrons to be transferred to unstable chemical species[40].

The antioxidant enzyme system, represented in this study by SOD, prevents the accumulation of oxygen and hydrogen peroxide, and thus is considered the main line of defense of the body[41]. CCl₄ significantly decreased SOD activity, and MLT reversed this process. Reduced SOD activity, along with increased LPO in animals from the CCl₄ group, establishes a situation of oxidative stress[41-43]. MLT protects hepatocytes against damage from free radicals by directly scavenging free radicals and stimulating antioxidant enzymes[44,45]. Similar to previous studies, our study found that MLT increased the activity of the antioxidant enzyme SOD, which occurred in parallel with Nrf2 activation [46,47].

Nrf2 is a major transcriptional regulator that secures a vast range of tissues and cells from ROS mediated induction because of its various antioxidant and phase II detoxification enzymes[4]. Furthermore, several chemopreventive compounds can abate tissue fibrosis by enhancing nuclear

translocation of Nrf2 and induction of glutathione S-transferases (GSTs) and SOD expression[4]. One of these essential mechanisms responsible for the induction of enzymes in response to stress is the pathway of Nrf2 and its inhibitor Keap1. When translocated into the nucleus, Nrf2 binds to the antioxidant responsive element (ARE), regulating the expression of endogenous antioxidants and proteins involved in the regulation of cell cycle and death[46-50]. In an Nrf2-knockout mouse model after long term CCl_4 treatment, the liver fibrosis was aggravated[4].

In this study, Nrf2 expression was significantly reduced, whereas Keap1 expression was greater in animals from the CCl_4 group. Studies have shown that Nrf2 plays a protective role in liver disease, with Nrf2-deficient rats being more sensitive and susceptible to liver injury and fibrosis induced by hepato-toxins[51]. Our study showed that the improvement in liver damage was due to MLT treatment. This improvement was due to MLT that is able to activate the nuclear factor Nrf2, resulting in less oxidative stress[51-54]. It has also been documented that Nrf2 activators, a wide variety of compounds such as sulfur-containing compounds, polyphenols, terpenoids, carotenoids, and selenium, exert the cytoprotective effect and emerge to play a part in the antioxidative response, presumably providing a series of protections needed for normal cellular activities. Furthermore, it is widely recognized that reduced Nrf2-mediated antioxidant defense plays a key role in the process of CCl_4-induced liver fibrosis. Nrf2 activators thus far exhibit hepatoprotective effects and significantly attenuate liver injury and fibrosis in clinical studies[4]. Kaufman *et al* in 2020 related that the modulation of the Nrf2 is dependent of zinc [55]. They evaluated cells incubated in zinc deficient medium and evidenced a reduction of the nuclear Nrf2 levels[55]. Zinc deficits aggravated the oxidative stress and reduced Nrf2 nuclear translocation[55-58].

As already observed in the study by Fernandes *et al*[59], cirrhotic animals showed significantly lower serum levels of zinc when compared to controls, showing an association of zinc deficiency with liver damage in sick animals.

Studies linking zinc and liver diseases often described that the replacement of the zinc element results in an improvement in the clinical and morphological condition[7,59]. We determined the zinc levels in the liver of rats exposed to CCl_4. Our results demonstrated reduced levels of zinc in injured livers. MLT treatment was effective in restoring normal zinc levels. A study with old rats treated with MLT showed a link between zinc and MLT. It is believed that MLT is able to modulate zinc turnover, due to the synchronization of circadian patterns of zinc and MLT and the concomitant increase in the levels of both in animals treated with MLT[56-58].

Our results show that MLT treatment in animals treated with CCl_4 resulted in an improvement in the liver enzyme pattern, lower oxidative stress, activation of Nrf2, and normalization of serum zinc levels. In addition to these findings, we measured the expression of ATF6 and GRP78/BiP, two important proteins involved in ER stress.

In the presence of stressors, GRP78/BiP is released, leading to the activation of the UPR signaling pathway, including ATF6[60]. Under the action of released GRP78/BiP, ATF6 decouples from the ER and undergoes cleavage in the Golgi system. At that moment, the activation of the nuclear factor (ATF6 50-kDa) that regulates the GRP78/ BiP and GRP94 proteins occurs, and the maintenance of this activation results in ER stress[61].

Physiological or pathological processes that disturb ER homeostasis led to a pathologic response called ER stress, causing the activation of the intracellular signaling pathway called UPR, thereby contributing to the pathogenesis of several conditions, including liver diseases[8,9,29,62]. A growing amount of evidence reinforces that increased ROS production is strongly related to induction of ER stress[6]. Our findings suggest the presence of ER stress in the animals from the CCl_4 group, since there was an increase in the expression of ATF6 and GRP78/BiP. The transcription of these chaperones is increased in response to various stimuli that disturb or overload ER function, including exposure to xenobiotics[29]. A significant decrease in the expression of proteins that predict ER stress was observed in animals receiving MLT. Our data are consistent with recent findings showing that MLT reduces ER stress in different models of cell injury[24]. For example, treatment with MLT reduced ER stress and modulated UPR in rabbits with fulminant hepatitis of viral origin, an effect that was associated with a reduction in apoptosis, cell death, and liver damage[24]. MLT also showed a neuroprotective effect through the reduction of ER stress in neuronal cells of newborn rats after hypoxia-ischemia[63]. Typically, ROS are controlled by intracellular antioxidants such as SOD; however, the excess demand in protein folding can overload antioxidant response. In support of this theory, the use of antioxidants has been shown to enhance protein folding and reduce apoptosis in response to ER stress[29].

Another highly regulatory mechanism essential for cellular redox homeostasis and protein folding involves HSPs and the nuclear factor HSF1[29]. Among HSPs, HSP70 is one of the protein families that has been more conserved in evolution, being expressed in the cell both constitutively and inductively. Our data show that animals exposed to CCl_4 showed higher expression of HSP70 and HSF1. One of the cellular responses to stress is HSP activation[64]. Several lines of evidence highlight the deleterious effects of HSPs on various human diseases, including cancer, in which case it promotes survival and proliferation of tumor cells and drug resistance[65]. Members of the HSP70 family have been particularly implicated in the pathophysiology and pathogenesis of several liver diseases such as hepatitis B and C, non-alcoholic steatohepatitis, autoimmune hepatitis, primary biliary cirrhosis, and others[26]. Treatment with MLT reduced HSP70 and HSF1 expression.

The exact mechanism leading to xenobiotic-induced cellular stress is still not well understood and may involve multiple factors, such as xenobiotic concentration, time of exposure, mechanism of action, cell type affected, among others. The combination of different cell damages, including oxidative stress, ER stress, UPR, and cytosolic responses, may lead to cell apoptosis[29].

CONCLUSION

We conclude that MLT acts as a potent antioxidant, promoting the activation of the nuclear factor Nrf2, which allows the reduction in oxidative stress. Concomitantly, we observed the restoration of serum zinc levels, which contributes to numerous hepatic cytoprotective processes. With the reduction of oxidative stress, it is possible to attenuate the ER stress and the unfolded protein response, as well as the cell damage caused by the toxic agent CCl_4.

The evaluation of new markers in this model may contribute to a better understanding of other pathophysiological mechanisms of cirrhosis and its complications. MLT may offer a promising therapeutic approach to liver diseases, given its effectiveness in the attenuation of oxidative stress. A major limitation in the application of MLT is that most of the studies were designed especially on rats. More research is needed in clinical trials to approve the potentials of this indolamine in humans.

ARTICLE HIGHLIGHTS

Research background

Chronic liver diseases are characterized by a multistep process that involves several molecules and cellular events to transform a normal parenchyma into a parenchyma with steatosis, increased collagen deposition, fibrosis, and cirrhosis. Melatonin (MLT) is a potent antioxidant molecule that is shown to have a beneficial effect in various pathological situations, due to its action against free radicals.

Research motivation

To reduce the generation of reactive oxygen species would be a way to slow the progression of cell damage observed in liver diseases.

Research objectives

To study the effects of MLT on biochemical analysis, on serum zinc levels, and on oxidative stress in rats exposed to carbon tetrachloride (CCl_4), and to evaluate the expression of proteins involved in cell damage, endoplasmic reticular stress, and unfolded protein response in animals with liver injury induced by CCl_4 and treated with MLT.

Research methods

Twenty male Wistar rats (230-250 g) were divided into four groups: Control rats, rats treated with MLT alone, rats treated with CCl_4 alone, and rats treated with CCl_4 plus MLT. CCl_4 was administered as follows: Ten doses every 5 d, ten every 4 d, and 7 every 3 d. MLT was administered intraperitoneally at a dose of 20 mg/kg from the 10th wk to the end of the experiment (16th wk).

Research results

Administration of CCl_4 caused an increase in liver enzyme levels, a reduction in serum zinc levels, and greater oxidative stress and ER stress. MLT treatment was able to reverse the changes promoted by the toxic agent CCl_4.

Research conclusions

We conclude that MLT acts as a potent antioxidant, promoting activation of the nuclear factor Nrf2, which allows the reduction in oxidative stress. Concomitantly, we observed the restoration of serum zinc levels, which contributes to numerous hepatic cytoprotective processes. With the reduction of oxidative stress, it is possible to attenuate the ER stress and the unfolded protein response, as well as the cell damage caused by the toxic agent CCl_4.

Research perspectives

MLT may offer a promising therapeutic approach to liver diseases, given its effectiveness in the attenuation of oxidative stress. A major limitation in the application of MLT is that most of the studies were designed especially on rats. More research is needed in clinical trials to approve the potentials of this indolamine in humans.

FOOTNOTES

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REFERENCES

1 **Friedman SL**. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008; **134**: 1655-1669 [PMID: 18471545 DOI: 10.1053/j.gastro.2008.03.003]
2 **Klaunig JE**, Kamendulis LM, Hocevar BA. Oxidative stress and oxidative damage in carcinogenesis. *Toxicol Pathol* 2010; **38**: 96-109 [PMID: 20019356 DOI: 10.1177/0192623309356453]
3 **Klaunig JE**, Wang Z, Pu X, Zhou S. Oxidative stress and oxidative damage in chemical carcinogenesis. *Toxicol Appl Pharmacol* 2011; **254**: 86-99 [PMID: 21296097 DOI: 10.1016/j.taap.2009.11.028]
4 **Gong Y**, Yang Y. Activation of Nrf2/AREs-mediated antioxidant signalling, and suppression of profibrotic TGF-β1/Smad3 pathway: a promising therapeutic strategy for hepatic fibrosis-A review. *Life Sci* 2020; **256**: 117909 [PMID: 32512009 DOI: 10.1016/j.lfs.2020.117909]
5 **Rashid K**, Sinha K, Sil PC. An update on oxidative stress-mediated organ pathophysiology. *Food Chem Toxicol* 2013; **62**: 584-600 [PMID: 24084033 DOI: 10.1016/j.fct.2013.09.026]
6 **Brookheart RT**, Michel CI, Listenberger LL, Ory DS, Schaffer JE. The non-coding RNA gadd7 is a regulator of lipid-induced oxidative and endoplasmic reticulum stress. *J Biol Chem* 2009; **284**: 7446-7454 [PMID: 19150982 DOI: 10.1074/jbc.M806209200]
7 **Grüngreiff K**, Reinhold D, Wedemeyer H. The role of zinc in liver cirrhosis. *Ann Hepatol* 2016; **15**: 7-16 [PMID: 26626635 DOI: 10.5604/16652681.1184191]
8 **Brenner C**, Galluzzi L, Kepp O, Kroemer G. Decoding cell death signals in liver inflammation. *J Hepatol* 2013; **59**: 583-594 [PMID: 23567086 DOI: 10.1016/j.jhep.2013.03.033]
9 **Lenna S**, Han R, Trojanowska M. Endoplasmic reticulum stress and endothelial dysfunction. *IUBMB Life* 2014; **66**: 530-537 [PMID: 25130181 DOI: 10.1002/iub.1292]
10 **Touitou Y**, Fevre-Montange M, Proust J, Klinger E, Nakache JP. Age- and sex-associated modification of plasma melatonin concentrations in man. Relationship to pathology, malignant or not, and autopsy findings. *Acta Endocrinol (Copenh)* 1985; **108**: 135-144 [PMID: 3969806 DOI: 10.1530/acta.0.1080135]
11 **Paredes SD**, Korkmaz A, Manchester LC, Tan DX, Reiter RJ. Phytomelatonin: a review. *J Exp Bot* 2009; **60**: 57-69

[PMID: 19033551 DOI: 10.1093/jxb/ern284]

12   **Reiter RJ**, Tan DX, Sainz RM, Mayo JC, Lopez-Burillo S. Melatonin: reducing the toxicity and increasing the efficacy of drugs. *J Pharm Pharmacol* 2002; **54**: 1299-1321 [PMID: 12396291 DOI: 10.1211/002235702760345374]

13   **Reiter RJ**, Tan DX, Terron MP, Flores LJ, Czarnocki Z. Melatonin and its metabolites: new findings regarding their production and their radical scavenging actions. *Acta Biochim Pol* 2007; **54**: 1-9 [PMID: 17351668]

14   **Wasser S**, Lim GY, Ong CN, Tan CE. Anti-oxidant ebselen causes the resolution of experimentally induced hepatic fibrosis in rats. *J Gastroenterol Hepatol* 2001; **16**: 1244-1253 [PMID: 11903743 DOI: 10.1046/j.1440-1746.2001.02621.x]

15   **Mills E**, Wu P, Seely D, Guyatt G. Melatonin in the treatment of cancer: a systematic review of randomized controlled trials and meta-analysis. *J Pineal Res* 2005; **39**: 360-366 [PMID: 16207291 DOI: 10.1111/j.1600-079X.2005.00258.x]

16   **Siegel D**, Bolton EM, Burr JA, Liebler DC, Ross D. The reduction of alpha-tocopherolquinone by human NAD(P)H: quinone oxidoreductase: the role of alpha-tocopherolhydroquinone as a cellular antioxidant. *Mol Pharmacol* 1997; **52**: 300-305 [PMID: 9271353 DOI: 10.1124/mol.52.2.300]

17   **Carbajo-Pescador S**, García-Palomo A, Martín-Renedo J, Piva M, González-Gallego J, Mauriz JL. Melatonin modulation of intracellular signaling pathways in hepatocarcinoma HepG2 cell line: role of the MT1 receptor. *J Pineal Res* 2011; **51**: 463-471 [PMID: 21718361 DOI: 10.1111/j.1600-079X.2011.00910.x]

18   **Pavanato A**, Tuñón MJ, Sánchez-Campos S, Marroni CA, Llesuy S, González-Gallego J, Marroni N. Effects of quercetin on liver damage in rats with carbon tetrachloride-induced cirrhosis. *Dig Dis Sci* 2003; **48**: 824-829 [PMID: 12741479 DOI: 10.1023/a:1022869716643]

19   **Cremonese RV**, Pereira-Filho AA, Magalhães R, de Mattos AA, Marroni CA, Zettler CG, Marroni NP. [Experimental cirrhosis induced by carbon tetrachloride inhalation: adaptation of the technique and evaluation of lipid peroxidation]. *Arq Gastroenterol* 2001; **38**: 40-47 [PMID: 11586997 DOI: 10.1590/s0004-28032001000100008]

20   **Rosa DP**, Bona S, Simonetto D, Zettler C, Marroni CA, Marroni NP. Melatonin protects the liver and erythrocytes against oxidative stress in cirrhotic rats. *Arq Gastroenterol* 2010; **47**: 72-78 [PMID: 20520979 DOI: 10.1590/s0004-28032010000100013]

21   **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-254 [PMID: 942051 DOI: 10.1006/abio.1976.9999]

22   **Buege JA**, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978; **52**: 302-310 [PMID: 672633 DOI: 10.1016/s0076-6879(78)52032-6]

23   **Misra HP**, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* 1972; **247**: 3170-3175 [PMID: 4623845]

24   **Tuñón MJ**, San-Miguel B, Crespo I, Laliena A, Vallejo D, Álvarez M, Prieto J, González-Gallego J. Melatonin treatment reduces endoplasmic reticulum stress and modulates the unfolded protein response in rabbits with lethal fulminant hepatitis of viral origin. *J Pineal Res* 2013; **55**: 221-228 [PMID: 23679826 DOI: 10.1111/jpi.12063]

25   **Mauriz JL**, Molpeceres V, García-Mediavilla MV, González P, Barrio JP, González-Gallego J. Melatonin prevents oxidative stress and changes in antioxidant enzyme expression and activity in the liver of aging rats. *J Pineal Res* 2007; **42**: 222-230 [PMID: 17349019 DOI: 10.1111/j.1600-079X.2006.00409.x]

26   **Liu T**, Daniels CK, Cao S. Comprehensive review on the HSC70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential. *Pharmacol Ther* 2012; **136**: 354-374 [PMID: 22960394 DOI: 10.1016/j.pharmthera.2012.08.014]

27   **Basu S**. Carbon tetrachloride-induced lipid peroxidation: eicosanoid formation and their regulation by antioxidant nutrients. *Toxicology* 2003; **189**: 113-127 [PMID: 12821287 DOI: 10.1016/s0300-483x(03)00157-4]

28   **El-Ansary AK**, Kotb M, Rizk MZ, Siddiqi NJ. Prooxidant mechanisms in toxicology. *Biomed Res Int* 2014; **2014**: 308625 [PMID: 24772419 DOI: 10.1155/2014/308625]

29   **Lafleur MA**, Stevens JL, Lawrence JW. Xenobiotic perturbation of ER stress and the unfolded protein response. *Toxicol Pathol* 2013; **41**: 235-262 [PMID: 23334697 DOI: 10.1177/0192623312470764]

30   **Mauriz JL**, Matilla B, Culebras JM, González P, González-Gallego J. Dietary glycine inhibits activation of nuclear factor kappa B and prevents liver injury in hemorrhagic shock in the rat. *Free Radic Biol Med* 2001; **31**: 1236-1244 [PMID: 11705702 DOI: 10.1016/s0891-5849(01)00716-x]

31   **Subramanian P**, Dakshayani KB, Pandi-Perumal SR, Trakht I, Cardinali DP. 24-hour rhythms in oxidative stress during hepatocarcinogenesis in rats: effect of melatonin or alpha-ketoglutarate. *Redox Rep* 2008; **13**: 78-86 [PMID: 18339250 DOI: 10.1179/135100008X259178]

32   **Srinivasan V**, Pandi-Perumal SR, Brzezinski A, Bhatnagar KP, Cardinali DP. Melatonin, immune function and cancer. *Recent Pat Endocr Metab Immune Drug Discov* 2011; **5**: 109-123 [PMID: 22074586 DOI: 10.2174/187221411799015408]

33   **Bona S**, Filippin LI, Di Naso FC, de David C, Valiatti B, Isoppo Schaun M, Xavier RM, Marroni NP. Effect of antioxidant treatment on fibrogenesis in rats with carbon tetrachloride-induced cirrhosis. *ISRN Gastroenterol* 2012; **2012**: 762920 [PMID: 22577570 DOI: 10.5402/2012/762920]

34   **de David C**, Rodrigues G, Bona S, Meurer L, González-Gallego J, Tuñón MJ, Marroni NP. Role of quercetin in preventing thioacetamide-induced liver injury in rats. *Toxicol Pathol* 2011; **39**: 949-957 [PMID: 21885874 DOI: 10.1177/0192623311418680]

35   **Arendt J**. Melatonin and the Mammalian Pineal Gland. London: Chapman & Hall, 1995: 331 [DOI: 10.14341/probl12060]

36   **Reiter RJ**, Tan DX, Manchester LC, Simopoulos AP, Maldonado MD, Flores LJ, Terron MP. Melatonin in edible plants (phytomelatonin): Identification, concentrations, bioavailability and proposed functions. *World Rev Nutr Diet* 2007; **97**: 211-230 [PMID: 17167296 DOI: 10.1159/000097917]

37   **Novo E**, Parola M. Redox mechanisms in hepatic chronic wound healing and fibrogenesis. *Fibrogenesis Tissue Repair* 2008; **1**: 5 [PMID: 19014652 DOI: 10.1186/1755-1536-1-5]

38   **Claustrat B**, Brun J, Chazot G. The basic physiology and pathophysiology of melatonin. *Sleep Med Rev* 2005; **9**: 11-24 [PMID: 15649735 DOI: 10.1016/j.smrv.2004.08.001]

39   **Anisimov VN**, Popovich IG, Zabezhinski MA, Anisimov SV, Vesnushkin GM, Vinogradova IA. Melatonin as antioxidant,

geroprotector and anticarcinogen. *Biochim Biophys Acta* 2006; **1757**: 573-589 [PMID: 16678784 DOI: 10.1016/j.bbabio.2006.03.012]

40   **Reiter RJ**, Tan DX, Manchester LC, Qi W. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. *Cell Biochem Biophys* 2001; **34**: 237-256 [PMID: 11898866 DOI: 10.1385/CBB:34:2:237]

41   **Wang H**, Wei W, Wang NP, Gui SY, Wu L, Sun WY, Xu SY. Melatonin ameliorates carbon tetrachloride-induced hepatic fibrogenesis in rats *via* inhibition of oxidative stress. *Life Sci* 2005; **77**: 1902-1915 [PMID: 15925388 DOI: 10.1016/j.lfs.2005.04.013]

42   **Oleshchuk O**, Ivankiv Y, Falfushynska H, Mudra A, Lisnychuk N. Hepatoprotective Effect of Melatonin in Toxic Liver Injury in Rats. *Medicina (Kaunas)* 2019; **55** [PMID: 31238587 DOI: 10.3390/medicina55060304]

43   **Halliwell B**. Free radicals and antioxidants: updating a personal view. *Nutr Rev* 2012; **70**: 257-265 [PMID: 22537212 DOI: 10.1111/j.1753-4887.2012.00476.x]

44   **Magierowski M**, Jasnos K, Pawlik M, Krzysiek-Maczka G, Ptak-Belowska A, Olszanecki R, Kwiecien S, Korbut R, and Brzozowski T The Involvement of Mas Receptor, Nitric Oxide, Prostaglandins, and Sensory Neuropeptides. J Pharmacol Exp Ther 2015; 353: 563 [PMID: 25934764 DOI: 10.1124/jpet.113.207233]

45   **Reiter RJ**, Rosales-Corral SA, Manchester LC, Liu X, Tan DX. Melatonin in the biliary tract and liver: health implications. *Curr Pharm Des* 2014; **20**: 4788-4801 [PMID: 24251672 DOI: 10.2174/1381612819666131119105826]

46   **Belmont-Díaz J**, López-Gordillo AP, Molina Garduño E, Serrano-García L, Coballase-Urrutia E, Cárdenas-Rodríguez N, Arellano-Aguilar O, Montero-Montoya RD. Micronuclei in bone marrow and liver in relation to hepatic metabolism and antioxidant response due to coexposure to chloroform, dichloromethane, and toluene in the rat model. *Biomed Res Int* 2014; **2014**: 425070 [PMID: 24949447 DOI: 10.1155/2014/425070]

47   **Jung KH**, Hong SW, Zheng HM, Lee DH, Hong SS. Melatonin downregulates nuclear erythroid 2-related factor 2 and nuclear factor-kappaB during prevention of oxidative liver injury in a dimethylnitrosamine model. *J Pineal Res* 2009; **47**: 173-183 [PMID: 19627459 DOI: 10.1111/j.1600-079X.2009.00698.x]

48   **Aleksunes LM**, Slitt AL, Maher JM, Dieter MZ, Knight TR, Goedken M, Cherrington NJ, Chan JY, Klaassen CD, Manautou JE. Nuclear factor-E2-related factor 2 expression in liver is critical for induction of NAD(P)H:quinone oxidoreductase 1 during cholestasis. *Cell Stress Chaperones* 2006; **11**: 356-363 [PMID: 17278884 DOI: 10.1379/csc-217.1]

49   **Kim HJ**, Vaziri ND. Contribution of impaired Nrf2-Keap1 pathway to oxidative stress and inflammation in chronic renal failure. *Am J Physiol Renal Physiol* 2010; **298**: F662-F671 [PMID: 20007347 DOI: 10.1152/ajprenal.00421.2009]

50   **Lee IC**, Kim SH, Baek HS, Moon C, Kang SS, Kim YB, Shin IS, Kim JC. The involvement of Nrf2 in the protective effects of diallyl disulfide on carbon tetrachloride-induced hepatic oxidative damage and inflammatory response in rats. *Food Chem Toxicol* 2014; **63**: 174-185 [PMID: 24246655 DOI: 10.1016/j.fct.2013.11.006]

51   **Li CM**, Li L, Wu J, Bai JY, Sun Y, Huang S, Wang GL. Upregulation of heat shock protein 32 with hemin alleviates acute heat-induced hepatic injury in mice. *Cell Stress Chaperones* 2014; **19**: 675-683 [PMID: 24473736 DOI: 10.1007/s12192-014-0495-6]

52   **Oh CJ**, Kim JY, Min AK, Park KG, Harris RA, Kim HJ, Lee IK. Sulforaphane attenuates hepatic fibrosis *via* NF-E2-related factor 2-mediated inhibition of transforming growth factor-β/Smad signaling. *Free Radic Biol Med* 2012; **52**: 671-682 [PMID: 22155056 DOI: 10.1016/j.freeradbiomed.2011.11.012]

53   **Farombi EO**, Shrotriya S, Na HK, Kim SH, Surh YJ. Curcumin attenuates dimethylnitrosamine-induced liver injury in rats through Nrf2-mediated induction of heme oxygenase-1. *Food Chem Toxicol* 2008; **46**: 1279-1287 [PMID: 18006204 DOI: 10.1016/j.fct.2007.09.095]

54   **Crespo I**, Miguel BS, Laliena A, Alvarez M, Culebras JM, González-Gallego J, Tuñón MJ. Melatonin prevents the decreased activity of antioxidant enzymes and activates nuclear erythroid 2-related factor 2 signaling in an animal model of fulminant hepatic failure of viral origin. *J Pineal Res* 2010; **49**: 193-200 [PMID: 20609075 DOI: 10.1111/j.1600-079X.2010.00787.x]

55   **Kaufman Z**, Salvador GA, Liu X, Oteiza PI. Zinc and the modulation of Nrf2 in human neuroblastoma cells. *Free Radic Biol Med* 2020; **155**: 1-9 [PMID: 32416241 DOI: 10.1016/j.freeradbiomed.2020.05.010]

56   **Lahiri DK**, Chen D, Lahiri P, Rogers JT, Greig NH, Bondy S. Melatonin, metals, and gene expression: implications in aging and neurodegenerative disorders. *Ann N Y Acad Sci* 2004; **1035**: 216-230 [PMID: 15681810 DOI: 10.1196/annals.1332.014]

57   **Mocchegiani E**, Santarelli L, Tibaldi A, Muzzioli M, Bulian D, Cipriano K, Olivieri F, Fabris N. Presence of links between zinc and melatonin during the circadian cycle in old mice: effects on thymic endocrine activity and on the survival. *J Neuroimmunol* 1998; **86**: 111-122 [PMID: 9663556 DOI: 10.1016/s0165-5728(97)00253-1]

58   **Mocchegiani E**, Bulian D, Santarelli L, Tibaldi A, Muzzioli M, Pierpaoli W, Fabris N. The immuno-reconstituting effect of melatonin or pineal grafting and its relation to zinc pool in aging mice. *J Neuroimmunol* 1994; **53**: 189-201 [PMID: 8071433 DOI: 10.1016/0165-5728(94)90029-9]

59   **Fernandes SA**, Bona S, Cerski CT, Marroni NP, Marroni CA. ALTERATION OF TASTE BUDS IN EXPERIMENTAL CIRRHOSIS. Is there correlation with human hypogeusia? *Arq Gastroenterol* 2016; **53**: 278-284 [PMID: 27706460 DOI: 10.1590/S0004-28032016000400013]

60   **Lee AS**. The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. *Methods* 2005; **35**: 373-381 [PMID: 15804610 DOI: 10.1016/j.ymeth.2004.10.010]

61   **Wang M**, Wey S, Zhang Y, Ye R, Lee AS. Role of the unfolded protein response regulator GRP78/BiP in development, cancer, and neurological disorders. *Antioxid Redox Signal* 2009; **11**: 2307-2316 [PMID: 19309259 DOI: 10.1089/ars.2009.2485]

62   **Zaouali MA**, Boncompagni E, Reiter RJ, Bejaoui M, Freitas I, Pantazi E, Folch-Puy E, Abdennebi HB, Garcia-Gil FA, Roselló-Catafau J. AMPK involvement in endoplasmic reticulum stress and autophagy modulation after fatty liver graft preservation: a role for melatonin and trimetazidine cocktail. *J Pineal Res* 2013; **55**: 65-78 [PMID: 23551302 DOI: 10.1111/jpi.12051]

63   **Carloni S**, Albertini MC, Galluzzi L, Buonocore G, Proietti F, Balduini W. Melatonin reduces endoplasmic reticulum stress and preserves sirtuin 1 expression in neuronal cells of newborn rats after hypoxia-ischemia. *J Pineal Res* 2014; **57**:

192-199 [PMID: 24980917 DOI: 10.1111/jpi.12156]

64   **Ashkenazi L**, Haim A. Light interference as a possible stressor altering HSP70 and its gene expression levels in brain and hepatic tissues of golden spiny mice. *J Exp Biol* 2012; **215**: 4034-4040 [PMID: 22933613 DOI: 10.1242/jeb.073429]

65   **Bellaye PS**, Burgy O, Causse S, Garrido C, Bonniaud P. Heat shock proteins in fibrosis and wound healing: good or evil? *Pharmacol Ther* 2014; **143**: 119-132 [PMID: 24582969 DOI: 10.1016/j.pharmthera.2014.02.009]

CASE REPORT

Hypereosinophilic syndrome presenting as eosinophilic gastroenteritis disorder and splinter hemorrhages: A case report

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Abstract

BACKGROUND
Eosinophilia and related organ damage are extensively studied hot topics among rare disorders. Any addition to the cohort of available case reports of the same will be adding knowledge for better management of this less known entity.

CASE SUMMARY
In this article, we describe a 27-year-old Indo-Aryan man who presented with abdominal pain, abdominal distension, and loose stools for variable days. He had splinter hemorrhages in the majority of fingernails. He was diagnosed with predominant eosinophilic gastrointestinal involvement with bowel obstruction and ascites, and was managed with intravenous immunoglobulin. He was subsequently treated with oral low dose steroid therapy and responded completely.

CONCLUSION
Our experience is evidence that prompt management of this hypereosinophilic lethal gastrointestinal (all three layers) infiltrative disease provides a cure and avoids complications. Splinter nail hemorrhages may be seen in the same disease.

Key Words: Ascites; Diarrhea; Eosinophilia; Intravenous immunoglobulin; Leucoproliferative disorder; Case report

Core Tip: This case reveals the wide spectrum of atypical manifestations of hyper-eosinophilic syndrome. Clinicians must be vigilant in order to diagnose primary instead of secondary eosinophilia as the former can cause diffuse organ infiltration and widespread tissue damage. Eosinophilic gastrointestinal disorder can present with involvement of all three layers of the gastrointestinal tract. Hypereosinophilic syndrome can also present with splinter hemorrhages. Prompt management of this lethal infiltrative disease provides a cure and avoids organ complications.

INTRODUCTION

Hypereosinophilic syndrome (HES) is an idiopathic, less defined, less known, primary leuco-proliferative disorder associated with peripheral blood eosinophilia and organ damage. The term HES was first used by Anderson[1] in 1968 in a case series, and since then, there have been considerable improvements in the understanding and hence changes in the definition and criteria for the diagnosis of this syndrome. The old definition (1975, Chusid *et al*[2]) included persistent blood eosinophilia ≥ 1.5 × 10^9/L lasting for > 6 mo, associated with eosinophil-mediated organ damage. However, the new definition (2010, Simon *et al*[3]) includes more than peripheral blood eosinophilia or duration of symptoms, and the evidence of eosinophil-mediated organ damage is enough for diagnosis of HES.

Presentations may vary, and can be organ-specific, may present as a myeloproliferative disorder, or may be asymptomatic. Treatment guidelines are not yet defined. Patients mostly present with organ damage in organ-specific eosinophilia syndrome and thus require an immediate intensive phase treatment to avoid organ failure and then a continuous phase of immunomodulator or steroids to avoid recurrences.

Eosinophilic gastroenteritis is a term reserved for an entity, where there is eosinophilic inflammation of more than one histologically distinct anatomic location in the gut[4]. Clinical presentation depends on the anatomical site and histological layer of the gut. The presentation can be as mild and chronic as dyspepsia, nausea, diarrhea, or as a differential for protein-losing enteropathy with mucosal layer involvement to as severe as crampy abdominal pain and intestinal obstruction with muscular layer involvement. The least common serosal type presents with eosinophilic ascites and abundant peripheral eosinophilia or rarely in severe cases as perforation[5].

Here we report a case of HES who presented with abdominal pain, distension, diarrhea, and splinter hemorrhages.

CASE PRESENTATION

Chief complaints
A 27-year-old Indo-Aryan man, resident of North-India, an electrician by profession, without addictions or co-morbidities presented with abdominal pain of 20 d, abdominal distension of 10 d, and loose stools of 3 d.

History of present illness
Abdominal pain was a continuous dull ache around the umbilicus. The pain became intermittent associated with the non-passage of flatus and feeling of fullness in the abdomen that was relieved with passage of flatus after some time. In the previous 10 d he had abdominal distension, which started around the left flank, and progressed to involve the whole abdomen. He had no vomiting, fever, jaundice, significant weight loss, urinary problems, chest problems, or lower limb swelling. Reports from outside our hospital showed eosinophilia (absolute count, 11400/μL).

History of past illness
He had no allergic tendency, pruritus ani, any recent intake of drugs, or B symptoms.

Personal and family history
No history of atopy in his family and no history suggestive of the same.
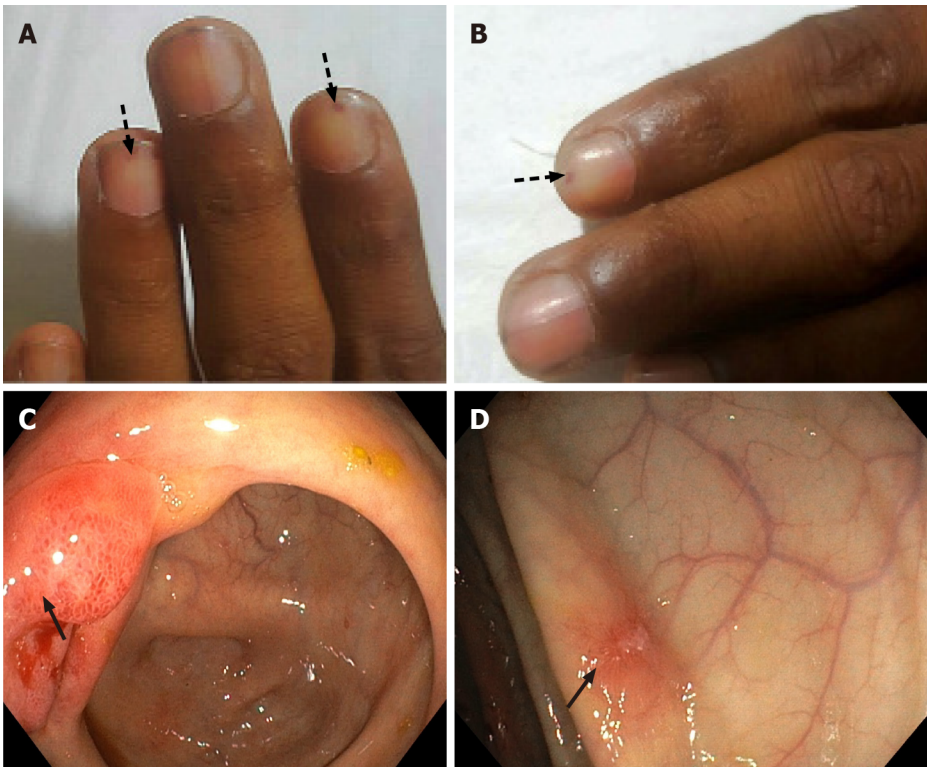
Figure 1 Cutaneous manifestations and endoscopic findings in the patient with eosinophilic gastrointestinal disorder. A and B: Sections show splinter hemorrhages (arrows) in different nails; C and D: Show terminal ileum and colonic mucosal erythematous erosions (arrows).

Physical examination

On examination, he was of average built (body mass index, 22 kg/m^2) with stable vitals. His local examination revealed splinter hemorrhages in digital nails (Figure 1A and B); however, fundoscopy did not show any Roth spots. Abdominal examination showed a distended abdomen with shifting dullness and sluggish bowel sounds without organomegaly or any stigmata of chronic liver disease. His systemic examination was non-contributory.

Laboratory examinations

His initial hemogram showed a leucocyte count of 33000/µL (absolute eosinophil count, 25000/µL) and normal hemoglobin and platelet count. Peripheral smear confirmed eosinophilic leukocytosis without any blasts or hemiparasites. Midnight diethyl carbamazine provocation test was negative (diethyl carbamazine single dose 300 mg at midnight to increase yield). His kidney and liver functions were essentially normal. Total IgE was 830 kUA/L (normal range, < 64 kUA/L). The ascitic fluid study revealed low SAAG ascites (ascitic albumin: 2.9 g/dL and SAAG: 0.7 g/dL) with raised leukocyte count (6000 cells/mm^3) and numerous eosinophils (95% polymorphs and 5% monomorphs) and reactive mesothelial cells. Multiple stool microscopy samples were obtained, however no evidence of parasites was observed.

Imaging examinations

Ultrasonography of the abdomen showed ascites with multiple echogenic foci (septated) and few subcentimetric mesenteric lymph nodes. ECG and chest X-ray were normal. A contrast-enhanced computed tomography (CT) of the abdomen was performed to rule out any occult abdominal pathology, which revealed diffuse bowel wall thickening and submucosal edema. Both upper gastrointestinal (GI) endoscopy and colonoscopy were performed and showed erythematous mucosa (Figure 1C and D). The biopsy from multiple sites (antrum, duodenum, ileum, cecum, rectum) revealed eosinophilic infiltration in the lamina propria with dense infiltration of muscularis mucosa at the terminal ileum (Figure 2). His esophageal biopsies were essentially normal.

FINAL DIAGNOSIS

At initial evaluation, differentials were parasitic infiltrations with secondary eosinophilia *vs* hyper-eosinophilic gastroenteritis *vs* hyper IgE syndrome. However, after all the investigations, the patient
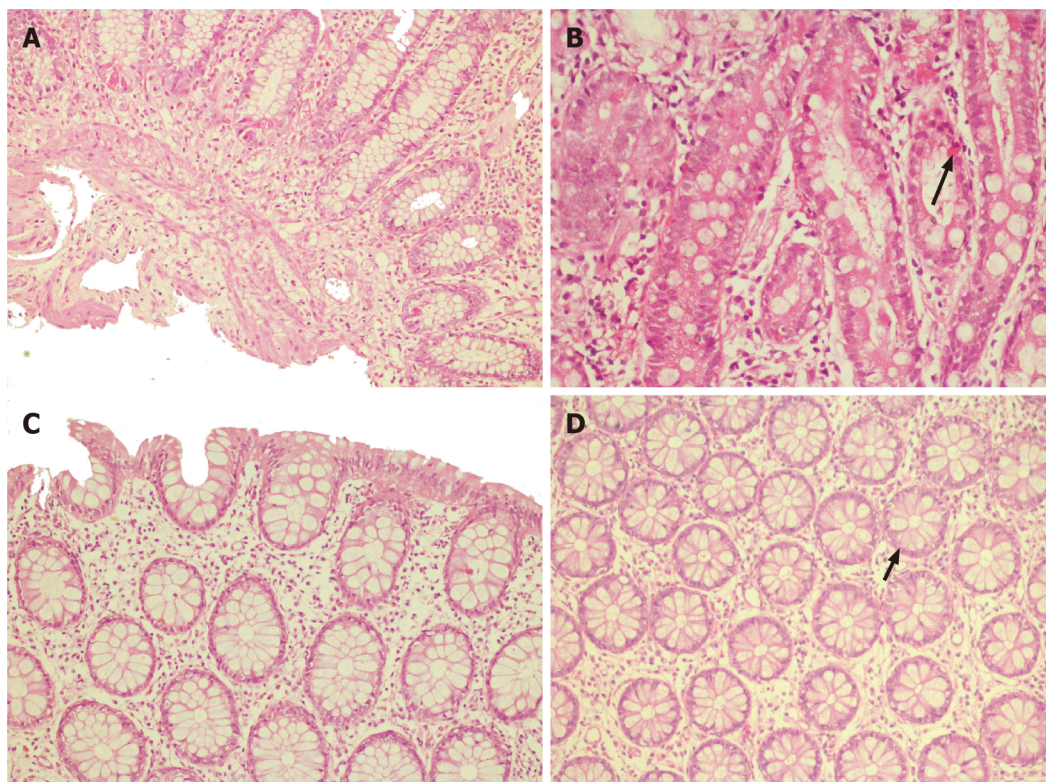
Figure 2 Histopathology images. A: Terminal ileal/ileocecal biopsy section shows mucosal lining with marked eosinophil rich infiltrates in the lamina propria. The eosinophils infiltrate the muscularis mucosae; B: Higher magnification (400×) of the duodenum section shows the infiltration of crypt epithelium by eosinophils (arrow); C: Section from the ascending colon lamina propria shows a mild increase in eosinophils; however, no cryptitis or crypt abscess is seen; D: Higher magnification (400×) of the rectum shows crypt lining epithelium infiltrated by eosinophils (arrow).

was confirmed to have hypereosinophilic syndrome presenting as eosinophilic gastroenteritis disorder (EGD) with atypical splinter hemorrhages.

TREATMENT

He was started on intravenous immunoglobulin (IVIG) at a total dose of 2 gm/kg divided over 5 d, after which he showed significant symptom improvement. He was started on a six food elimination diet (cow's milk, soy, wheat, egg, peanut, seafood/shellfish). His abdominal girth had reduced from 96 cm to 81 cm at discharge. His eosinophilic count also reduced to 1200/µL.

He was discharged on oral prednisolone 30 mg/d and planned to taper off.

OUTCOME AND FOLLOW-UP

In his last follow-up after 2-mo, there was no evidence of ascites or splinter hemorrhages. He has resumed work with normal eosinophil counts on low-dose steroid (prednisolone 10 mg/d) and plans to stop the steroid after 6-mo with a wait and watch policy.

DISCUSSION

This particular case revealed two major findings concerning HES. One is EGD and the other is splinter hemorrhages. EGD is proposed to have a centrifugal eosinophilic infiltrate starting from the mucosa to serosa evidenced by the fact that the disease with serosal and muscular types always has mucosal involvement[6]. This case had symptoms suggestive of the involvement of all the three layers of the GI tract: Diarrhea and hypoalbuminemia suggestive of mucosal involvement, intestinal obstruction, and related symptoms suggestive of muscular involvement, and exudative ascites suggestive of serosal involvement. Such extensive symptomatology, whether reported in a single case or not is to our knowledge unknown. The classification or therapeutic guideline for HES is still limited as most review
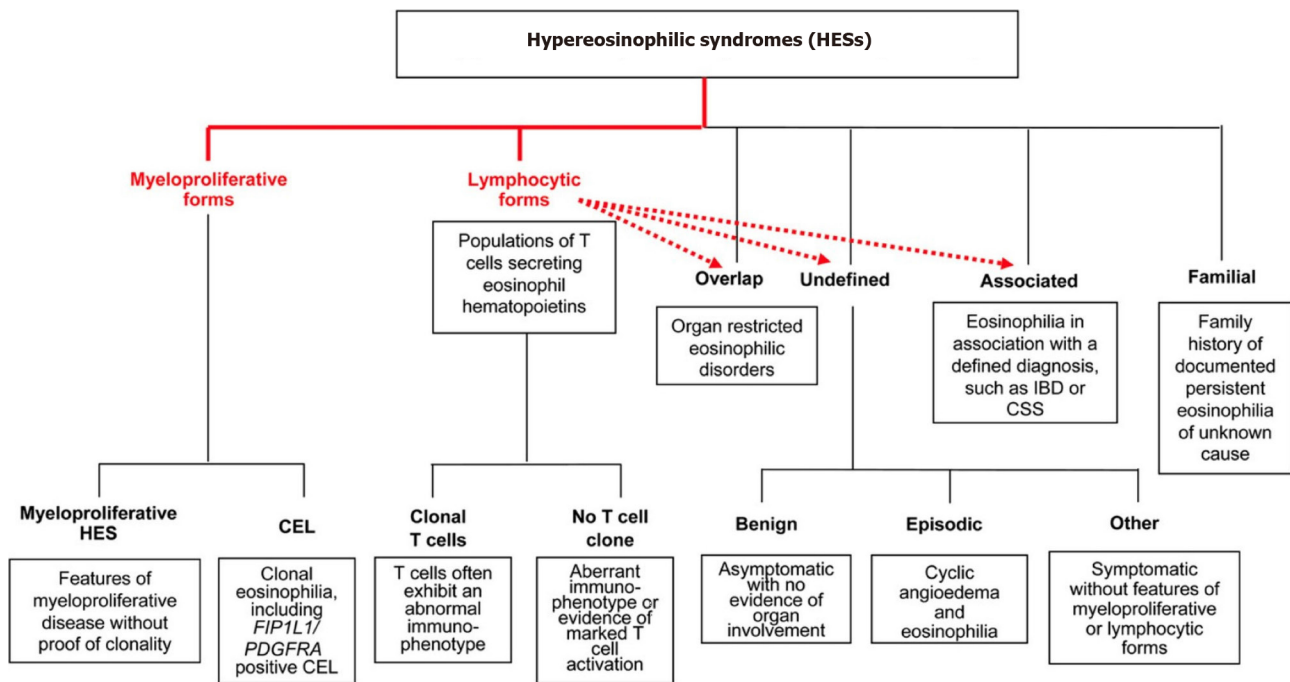
Figure 3 The classification of hypereosinophilic syndrome as per Simon *et al*[3]. CSS: Cyberchondria severity scale; IBD: Inflammatory bowel disease; HES: Hypereosinophilic syndrome; CEL: Carboxyl ester lipase. Citation: Simon HU, Rothenberg ME, Bochner BS, Weller PF, Wardlaw AJ, Wechsler ME, Rosenwasser LJ, Roufosse F, Gleich GJ, Klion AD. Refining the definition of hypereosinophilic syndrome. *J Allergy Clin Immunol* 2010; 126: 45-49.

articles are based on case reports and case series. To date, no randomized studies have been carried out in the adult population regarding management. Simon *et al*[3] classified HES into multiple independent categories based on clinical manifestations and pathogenesis (Figure 3). Lymphoproliferative HES is associated with increased production of eosinophil hematopoietin (mainly IL-5) by activated T-lymphocytes[7]. Another feature associated with the same is increased levels of serum IgE, dermatologic manifestations, and gastrointestinal symptoms. Hence our case could be a type of this variety. Other types such as undefined and organ-specific variants are also associated with a similar pathogenesis, lymphocyte-mediated increased eosinophil hematopoietin production, and related eosinophilic infiltration and organ damage[8]. These variants are responsive to immunosuppressive therapy, both to steroids and steroid-sparing immunomodulatory agents.

Eosinophilic gastroenteritis was first reported in 1937; however, the pathogenesis and management are unknown. There are two main reasons for this apparent contradiction. Firstly, it presents just like any other GI disorder with heterogeneous and nonspecific symptoms and is thus under-diagnosed. Secondly, it is a less prevalent disease[4]. The pathogenesis of EGD is complex and multifactorial. A trigger factor such as a food allergen, helminth, or bacterial infection is often involved, leading to and IL-5 mediated eosinophilic recruitment to the GI tract. The eosinophil-mast cell axis is a known factor involved in functional GI disorder[4]. Activated mast cells secrete histamine and serotonin, which results in visceral sensitivity and motility disorder. The same axis is proposed to function in EGD. EGD can be associated with or without peripheral eosinophilia and diagnosis thus remains tricky. In the presence of ascites, a diagnostic tap showing exudative ascites with eosinophilia is suggestive of the disease[9]. Endoscopy guided biopsy is a useful test; however, patchy involvement of the GI tract means that diagnosis can be missed in 25% of cases[10]. In such cases, CT imaging is useful to identify thickened bowel areas where a surgical biopsy can be attempted[11]. Small bowel MRI, as well as CT scanning, are nonspecific in diagnosing eosinophilic gastroenteritis and use of imaging is to guide biopsies. Presently, no studies comparing the diagnostic accuracy of these procedures are available. Capsule endoscopy[12] may be used to detect eosinophilic gastroenteritis as it may provide clues for lesions in the small bowel which may be difficult to diagnose with conventional endoscopy; however, studies pertaining to the same are not available.

Subungual splinter hemorrhages are vertical hemorrhages occurring in the capillary loops of the nail. Their association with an eosinophilic syndrome was provided in a case report of eosinophilic endomyocarditis[13]. Their association with HES or EGD has not been reported to date. The pathogenesis is questionable as cutaneous manifestations of HES are related to dermal microthrombosis. Usual cutaneous manifestations are angioedema, erythematous pruritic papules and nodules, generalized erythroderma, and blistering skin lesions in the form of vesiculobullous lesions[14]. In infective endocarditis, splinter hemorrhages are thought to be due to the embolic phenomenon and subsequent hemorrhagic changes[15]. Thus, hemorrhagic changes on microthrombosis can be the reason for splinter hemorrhages in HES but further research and clarification are needed.

EGD is still a novel disease requiring extensive research for further knowledge expansion. Until then treatment is based primarily on clinician experience. Treatment strategies involve immunosuppression with corticosteroids, leukotriene receptor antagonists, mast cell stabilizers, antihistamines, or treatment with immunomodulators such as 6-mercaptopurine and azathioprine. Novel therapies include biologicals like Reslizumab (IL5 antagonist), Infliximab (TNF alpha inhibitor), Omalizumab (a monoclonal antibody against IgE), or IVIG[4]. Our patient presented with alarming symptoms of bowel obstruction and exudative ascites, hence we preferred to start him on IVIG therapy, to which he responded, potentially avoiding surgical intervention, and then started him on oral prednisolone therapy. Hence, we suggest the use of IVIG therapy in patients with alarming symptoms for rapid response. However, this was a single case report and hence requires blinded, prospective and randomized studies to determine the efficacy of IVIG for HES.

CONCLUSION

Our experience provides evidence that prompt management of this infiltrative disease offers a cure and avoids organ complications. Clinicians must be vigilant in order to diagnose primary instead of secondary eosinophilia as the former can cause diffuse organ infiltration and widespread tissue damage. EGD can present with the involvement of all three layers of the GI tract. HES can also present with splinter hemorrhages.

FOOTNOTES

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REFERENCES

1   **Anderson RE**. The hypereosinophilic syndromes. *Aun Intern Med* 1968; **68**: 1220

2   **Chusid MJ**, Dale DC, West BC, Wolff SM. The hypereosinophilic syndrome: analysis of fourteen cases with review of the literature. *Medicine (Baltimore)* 1975; **54**: 1-27 [PMID: 1090795]

3   **Simon HU**, Rothenberg ME, Bochner BS, Weller PF, Wardlaw AJ, Wechsler ME, Rosenwasser LJ, Roufosse F, Gleich GJ, Klion AD. Refining the definition of hypereosinophilic syndrome. *J Allergy Clin Immunol* 2010; **126**: 45-49 [PMID: 20639008 DOI: 10.1016/j.jaci.2010.03.042]

4   **Sunkara T**, Rawla P, Yarlagadda KS, Gaduputi V. Eosinophilic gastroenteritis: diagnosis and clinical perspectives. *Clin Exp Gastroenterol* 2019; **12**: 239-253 [PMID: 31239747 DOI: 10.2147/CEG.S173130]

5   **Chen MJ**, Chu CH, Lin SC, Shih SC, Wang TE. Eosinophilic gastroenteritis: clinical experience with 15 patients. *World J Gastroenterol* 2003; **9**: 2813-2816 [PMID: 14669340 DOI: 10.3748/wjg.v9.i12.2813]

6   **Chang JY**, Choung RS, Lee RM, Locke GR 3rd, Schleck CD, Zinsmeister AR, Smyrk TC, Talley NJ. A shift in the clinical spectrum of eosinophilic gastroenteritis toward the mucosal disease type. *Clin Gastroenterol Hepatol* 2010; **8**: 669-75; quiz e88 [PMID: 20451664 DOI: 10.1016/j.cgh.2010.04.022]

7   **Roufosse F**, Cogan E, Goldman M. Lymphocytic variant hypereosinophilic syndromes. *Immunol Allergy Clin North Am* 2007; **27**: 389-413 [PMID: 17868856 DOI: 10.1016/j.iac.2007.07.002]

8   **Simon D**, Simon HU. Eosinophilic disorders. *J Allergy Clin Immunol* 2007; **119**: 1291-300; quiz 1301 [PMID: 17399779 DOI: 10.1016/j.jaci.2007.02.010]

9   **Harmon WA**, Helman CA. Eosinophilic gastroenteritis and ascites. *J Clin Gastroenterol* 1981; **3**: 371-373 [PMID: 7338618 DOI: 10.1097/00004836-198112000-00013]

10   **Talley NJ**, Shorter RG, Phillips SF, Zinsmeister AR. Eosinophilic gastroenteritis: a clinicopathological study of patients with disease of the mucosa, muscle layer, and subserosal tissues. *Gut* 1990; **31**: 54-58 [PMID: 2318432 DOI: 10.1136/gut.31.1.54]

11   **Wiesner W**, Kocher T, Heim M, Bongartz G. CT findings in eosinophilic enterocolitis with predominantly serosal and muscular bowel wall infiltration. *JBR-BTR* 2002; **85**: 4-6 [PMID: 11936478]

12   **Herrera Quiñones G**, Scharrer SI, Jiménez Rodríguez AR, García Compean D, Borjas Almaguer OD, Martínez Segura JA, Jáquez Quintana JO, González González JA, Maldonado Garza HJ. Diagnosis of Eosinophilic Enteritis With Video Capsule Endoscopy and Double Balloon Enteroscopy With Favorable Response to Corticosteroids. *ACG Case Rep J* 2019; **6**: e00127 [PMID: 31620526 DOI: 10.14309/crj.0000000000000127]

13   **Usui S**, Dainichi T, Kitoh A, Miyachi Y, Kabashima K. Janeway Lesions and Splinter Hemorrhages in a Patient With Eosinophilic Endomyocarditis. *JAMA Dermatol* 2015; **151**: 907-908 [PMID: 25924060 DOI: 10.1001/jamadermatol.2015.0388]

14   **Weller PF**, Bubley GJ. The idiopathic hypereosinophilic syndrome. *Blood* 1994; **83**: 2759-2779 [PMID: 8180373]

15   **Kilpatrick ZM**, Greenberg PA, Sanford JP. Splinter hemorrhages--their clinical significance. *Arch Intern Med* 1965; **115**: 730-735 [PMID: 14299446 DOI: 10.1001/archinte.1960.03860180102019]