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Research Article

# Melatonin Aggravated Oxaliplatin-Mimicking Sinusoidal Obstruction Syndrome: Role of Platelet Aggregation and Oxidative Stress

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**Abstract**

***Background*:** Sinusoidal obstruction syndrome is caused by an injury to the liver induced either by accidental ingestion of pyrrolizidine alkaloid monocrotaline or by chemotherapeutic drug oxaliplatin used for the treatment of colorectal cancer. Sinusoidal obstruction syndrome is characterized by rounding and swelling of the sinusoidal endothelial cell, which leads to obstruction of blood vessels, leukocyte infiltration, and oxidative stress. Melatonin, a powerful antioxidant, prevents acute liver injury. We investigated the effect of melatonin on monocrotaline-induced sinusoidal obstruction syndrome in mice.

***Methods*:** Male C57BL/6JNarl mice were injected with a single dose (500 mg/kg) of monocrotaline to induce sinusoidal obstruction syndrome. Melatonin (1-30 mg/kg) was injected 1 h before monocrotaline treatment. After 24 h of monocrotaline treatment, mice were sacrificed. Hepatic collagen, oxidative stress, antioxidant activity, and the expression of apoptosis protein were measured.

***Results*:** Melatonin aggravated monocrotaline-induced sinusoidal obstruction syndrome. In addition, it not only increased serum glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, neutrophil count, histological score, hepatic platelet aggregation, oxidative stress, and activated the apoptosis signalling pathway, but also decreased the red blood cell count, hematocrit ratio, platelet count, lymphocyte count, hepatic collagen, and antioxidant activity.

***Conclusions*:** Melatonin aggravated monocrotaline-induced sinusoidal obstruction syndrome via hepatic platelet aggregation and oxidative stress, ultimately leading to activation of apoptosis.

**Keywords**

Melatonin; monocrotaline; sinusoidal obstruction syndrome; oxaliplatin; liver injury

## 1. Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a pineal neurohormone, predominantly secreted and released in response to darkness at night in mammals and humans. Melatonin plays a pivotal role in the regulation of the biological clock, specifically the sleep-wake cycle and the stimulation of physiological sleep. In addition, it is a regulator of circadian rhythms and alleviates insomnia and jet lag [1, 2]. Furthermore, it is a well-known powerful antioxidant [3]. It protects against oxidative stress by eliminating ROS, enhances antioxidant enzyme activity, and preserves the antioxidant glutathione [4]. Melatonin inhibits hepatic dysfunction by attenuating myeloperoxidase (MPO), serum glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT) [5]. Melatonin attenuates sinusoidal dilatation, apoptosis, nitric oxide, inflammatory cell infiltration, and hepatic necrosis [6, 7]. In addition, melatonin regulates various key physiological functions such as immune function, energy metabolism, the cardiovascular system, reproductive system, and neuropsychiatric system [8, 9]. Melatonin exhibits anticancer activity by inhibiting genomic instability, sustained proliferative signals, replicative mortality, dysregulated metabolism, tumour-promoting inflammation, angiogenesis, metastasis, and immune evasion. In addition, melatonin stimulates anti-growth signalling and apoptosis [10, 11]. Melatonin is also known to possess few adverse effects such as headache, somnolence, palpitations, abdominal pain, dizziness, nausea, vomiting, and nightmares [12]. Melatonin should be avoided in a few cases such as renal impairment, alcohol addiction, high lipid levels, pregnancy, and blood coagulation dysfunction [13].

Oxaliplatin, a third-generation platinum analogue, acts through interruption of DNA synthesis, and is an efficient chemotherapeutic drug for various solid tumours, including stage IV colorectal and stomach cancer. Oxaliplatin is the commonly recommended and used chemotherapeutic agent for hyperthermic intraperitoneal chemotherapy [14]. The first clinical study in 2004 reported severe oxaliplatin-induced hepatic sinusoidal obstruction (SOS) (Rubbia-Brandt et al., 2004). Since then, oxaliplatin-induced SOS was a major issue in patients receiving hepatic resection for metastatic colorectal cancer. The incidence of oxaliplatin-induced SOS in metastatic colorectal cancer is up to 78% [15, 16]. SOS, also known as hepatic veno-occlusive disease, is

described as sinusoidal dilatation, the rounding up of sinusoidal endothelial cells with erythrocyte congestion and extravasation in centrilobular hepatic zones such as the central and portal veins. In addition, SOS causes neutrophil recruitment, platelet aggregation, depletion of glutathione, promotion of inflammation, and oxidative stress [17]. Oxaliplatin-induced SOS is characterized by the breakdown of the sinusoidal membrane, and sinusoidal dilatation with red blood cell congestion in the central vein area due to damage to the sinusoidal endothelial cells and lining [18, 19]. In addition, oxaliplatin manifests other adverse effects such as insomnia, hematologic effects, vomiting, diarrhoea, peripheral neuropathy, and pulmonary toxicity, mainly characterized by interstitial pneumonitis or bronchiolitis obliterans organizing pneumonia [14, 19].

Ingestion of monocrotaline, a pyrrolizidine alkaloid, via dietary sources is known to cause SOS. Acute monocrotaline-induced SOS is analogous to human SOS and characterized by elevated levels of aspartate transaminase (AST), alanine transaminase (ALT), hepatic injury, neutrophils, and myeloperoxidase (MPO) activity [18, 20, 21]. Monocrotaline-induces SOS via the apoptotic mechanism of injury leading to the damage of endothelial cells in the liver followed by hepatocyte apoptosis and extrusion into sinusoids, causing obstruction and congestion of sinusoids [22]. Monocrotaline induces high levels of ROS, subsequently depleting antioxidants which leads to cell death by apoptosis. Furthermore, thioredoxin directly inhibits proapoptotic proteins such as apoptosis signal-regulating kinase 1 (ASK1). Thus, inhibition of thioredoxin results in activation of ASK1 and induction of apoptosis [23], leading to acute SOS.

In monocrotaline-mimicking oxaliplatin-induced SOS, ROS and sinusoidal dilation play a major factor in inducing SOS. In addition, clinically, oxaliplatin chemotherapy manifests insomnia. Melatonin is a well-known antioxidant and sleep medicine that may prevent SOS and treat insomnia. The aim of this study was to investigate the protective effect of melatonin on monocrotaline-induced SOS in mice.

## 2. Materials and Methods

### *2.1 Materials*

Melatonin was bought from Tokyo Chemical Industry, Japan. Monocrotaline was purchased from Sigma Chemicals, USA. All other chemicals used were research grade. The animal care and experimental protocols were in accordance with nationally approved guidelines. Mice were housed in an environmentally controlled animal facility on a 12:12 light/dark cycle with food and water available ad libitum in the laboratory animal section. All animal experiments were approved by the Ethics Committee at National Cheng Kung University. Mice were anaesthetized by using Zoletil-50 (Virbac, Carros, France) (50 mg/kg, i.p.) and sacrificed by using $CO_2$ inhalation at the end of the experiments.

### *2.2 Animals*

Male C57BL/6Narl (B6) mice 8 weeks old and weighing 20 ± 2 g were obtained from the National Laboratory Animal Center and housed in the National Cheng Kung University Animal Center. The mice were given pellet feed (Richmond Standard; PMI Feeds, Inc., St. Louis, MO) and water ad libitum. They had a 12-h light/dark cycle and central air conditioning (25°C, 70% humidity)

throughout the experimental period. The animal care and experimental protocols were in accordance with nationally approved guidelines.

Monocrotaline (10 mg/mL) solution was prepared, by dissolving 500 mg of monocrotaline is dissolved in 2 N HCl and the pH is adjusted to 7.4 with 4 M NaOH phosphate-buffered saline (PBS), which is added to increase the total volume to 50 mL [21].

### *2.3 Preparation of Melatonin*

Melatonin solution was prepared fresh before injection by dissolving melatonin in absolute ethanol and further diluted with normal saline; the final concentration of ethanol is 2% [24].

### *2.4 Experimental Design*

#### 2.4.1 Monocrotaline-Induced SOS

SOS was experimentally induced by intraperitoneal administration of MCT to mice. The mice fasted for 12 h before MCT administration, but they were allowed water ad libitum. MCT (500 mg/kg) was administered by intraperitoneal injection and then the mice were allowed food and water ad libitum.

#### 2.4.2 Experimental Protocol

Experiment 1: Dosage fixation. The mice were divided into seven groups (n = 6). In Group I, the mice were treated only with saline. In Group II, the mice were injected with only monocrotaline (500 mg/kg). Groups III, IV, V, and VI were first given different doses of melatonin (1, 3, 10, and 30 mg/kg, respectively) and 1 h later, monocrotaline (500 mg/kg) via intraperitoneal injection. In Group VII, mice were injected only with melatonin (30 mg/kg). After 24 h, the mice were sacrificed. The blood and liver were collected from each mouse. Organ damage was evaluated by serum biochemistry, haematology analysis, and histological examination. Serum glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were measured.

Experiment 2: Analysis of liver oxidative stress. The mice were divided into four groups (n = 6). In Group I, mice were treated only with saline. In Group II, mice were injected only with monocrotaline (500 mg/kg). Group III's mice were first given melatonin (30 mg/kg) and 1 h later intraperitoneally injected with monocrotaline (500 mg/kg). The mice in Group IV were injected only with melatonin (30 mg/kg). After 24 h, all mice were sacrificed. The blood and liver were collected. Liver malondialdehyde (MDA), nitric oxide (NO), glutathione (GSH), and thioredoxin Reductase (TrxR) activity were measured.

### *2.5. Methods*

#### 2.5.1 Blood Collection

Blood samples were collected in serum separation tubes from the heart while the mice were under mild anaesthesia. The tubes were allowed to clot for 30 min at room temperature and then centrifuged at 15,000 rpm at 4°C for 20 min.

2.5.2 Assessing SOS

Hepatic injury by measured by identifying the levels of GOT and GPT in serum using a biochemistry analyzer (Dri-Chem 3500s; Fujifilm, Kanagawa, Japan). Hepatic injury was further confirmed using histological studies. A small piece of liver tissue was cut from each mouse and placed in 10% phosphate-buffered formalin. The tissue pieces were dehydrated using a graded percentage of alcohol and then fixed in paraffin wax for 1 h to form blocks. The blocks were trimmed and cut into 4-µm-thick sections, stained with hematoxylin and eosin, and then mounted; the sections of liver tissue were examined under a microscope (BX51; Olympus America, 100x) to assess hepatic injury. To quantify the degree of SOS, we used Image J software (National Institute of Health, USA). Briefly, the stained liver sections were photographed, and 8 photomicrographs were randomly selected for further analysis. These photomicrographs were converted to grayscale and split into red, green, and blue channels. We used the green channel, which has the best contrast. A threshold level of 87 was set to all the photomicrographs. The percentage of threshold area were measured and then converted to score. The score was calculated on a 6-point scale using the percentage of threshold area: 0 = 0-5%; 1 = 6-10%; 2 = 11-15%; 3 = 16-20%; 4 = 21-25%; 5 = 26-30%; 6 = 31-35%; 7 = 36-40%; 8 = 41-45%; 9 = 46-50%; 10 = 51-55%.

2.5.3 Serum Biochemical Analyses

GOT and GPT were used to assess liver function. Fifteen µL of centrifuged serum samples were analyzed with a blood biochemical analyzer (Fujifilm DRII-CHEM 3500s; Fujifilm, Kanagawa, Japan).

2.5.4 Haematology Analyses

Red blood cell count (RBC), hematocrit ratio (HCT), platelet count, lymphocyte count, and neutrophil count were checked in the blood to assess liver function. One hundred µL of blood was added into EDTA and blood count was analyzed by a haematology analyzer (Scil Vet Focus 5).

2.5.5 Masson Trichrome Staining for Collagen

Tissue sections were deparaffinized, rehydrated, and then Masson trichrome stained. Random pictures were taken at different locations of the tissue section under 10 high-power fields (100x). Scoring was done around the central vein and portal vein.

2.5.6 Measuring Liver Lipid Peroxidation

A 10% liver-tissue homogenate (0.5 g in 0.5 mL of MDA assay buffer) was used to measure LPO levels. In brief, tissue samples were homogenized and centrifuged (15,000 rpm at 4°C for 20 min), and the supernatant (250 µL) was taken to measure liver LPO using a NWLSSTM Malondialdehyde Assay Kit (Northwest Life Science Specialties, Vancouver, WA, USA) and read with a spectrophotometer (DU 640B; Beckman, Fullerton, CA, USA) at 532 nm.

2.5.7 Measuring Liver Nitric Oxide Levels

A 10% liver-tissue homogenate (0.3 g in 0.3 mL of deionized water) was used to measure NO levels. In brief, tissue samples were homogenized and centrifuged (15,000 rpm at 4°C for 20 min).

The supernatant (100 µL) was incubated with 100 µL of Griess reagent at room temperature for 20 min, and read with a spectrophotometer (DU 640B; Beckman, Fullerton, CA, USA) at 550 nm.

2.5.8 Measuring Liver Glutathione Levels

A 10% liver-tissue homogenate (0.8 g in 0.8 mL of ice-cold 10% trichloroacetic acid) was used to measure GSH levels. In brief, tissue samples were homogenized and centrifuged (15,000 rpm at 4°C for 20 min), and then 500 µL of the supernatant was added to 2 mL of 0.3 M $Na_2HPO_4 \cdot 2H_2O$ solution. Next, 200 µL dithiobis (2-nitrobenzoic acid in 1% sodium citrate, 0.4 mg/mL) was added and the absorbance was immediately measured at 412 nm.

2.5.9 Measuring Liver Thioredoxin Reductase Activity

A 10% liver-tissue homogenate (0.2 g in 0.2 mL of cold buffer) was used to measure TrxR levels. In brief, tissue samples were homogenized and centrifuged (10,000 rpm at 4°C for 15 min), and the supernatant (40 µL) was taken to measure liver thioredoxin reductase levels using a Thioredoxin Reductase Colorimetric Assay Kit (Cayman, USA). The absorbance was immediately measured at 405 nm.

2.5.10 Western Blot Analysis

We loaded 100 µg of protein on 10% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis and then transferred it to nitrocellulose sheets (NEN Life Science Products, Inc., Boston, MA, USA) in a transfer apparatus (Bio-Rad). The transfer was done by applying 1.2 A current for 3 h. After the blots had been blocked in 5% nonfat skim milk in Tris-buffered saline-Tween-20, we incubated the blots with primary iNOS, NF-κB, Trx, TNXRD1, Bax, and Caspase 3 antibody against the target protein in 5% nonfat skim milk and then with anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (dilution, 1: 500-1000; Jackson ImmunoResearch Laboratories, Inc., Philadelphia, PA, USA). Immunoblots were developed using alkaline phosphatase substrate solution (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium; Kirkegaard and Perry Laboratories, Inc., Baltimore, MD, USA).

2.5.11 Immunohistochemical (IHC) Staining of Thioredoxin

The liver tissue sections were deparaffinized, rehydrated, blocked, and incubated with thioredoxin (dilution 1:200; Peoteintech, USA) overnight at 4°C and then developed using the Ultra Vision Detection System Anti-Rabbit, HRP/DAB, Ready-to-use Kit (Thermo Fisher). The stained sections were counterstained with hematoxylin. After being mounted using DPX, the slides were examined under a microscope (at 40x magnification).

2.5.12 Immunofluorescence of CD41 and ASK1 Expression

The liver tissue sections were deparaffinized, rehydrated, blocked, and then incubated with Anti-CD41 and Anti-ASK1 (dilution, 1:100; Abcam, Cambridge, UK) overnight at 4°C in the dark. After washing, the sections were incubated with a secondary antibody, goat anti-mouse IgG H&L (Alexa Fluor®647) (dilution, 1:200; Abcam, Cambridge, UK). The stained sections were examined

after using the Fluroshield mounting medium with 4', 6-diamidino-2-phenylindole (DAPI) (Abcam) under a fluorescent microscope (magnification, 100x) in the dark.

### *2.6 Statistical Analysis*

Data were expressed as mean ± standard deviation (SD). Significant differences of measurement traits were analyzed using one-way analysis of variance (ANOVA) followed by Student's t-test analysis. The significance was set at $p < 0.05$.

## 3. Results

### *3.1 Melatonin Exacerbated Hepatic Dysfunction in SOS Mice*

To assess the hepatoprotective effects of melatonin in monocrotaline-induced SOS, serum GOT and GPT levels were quantified. GOT (Figure 1A) and GPT (Figure 1B) levels were significantly increased in Group II animals compared to Group I. However, the level of GOT and GPT did not increase in Group III and IV animals compared to Group II. The level of GOT and GPT significantly increased in Group V and VI compared to Group II. GOT and GPT did not show any alterations in Group VII animals compared to Group I.

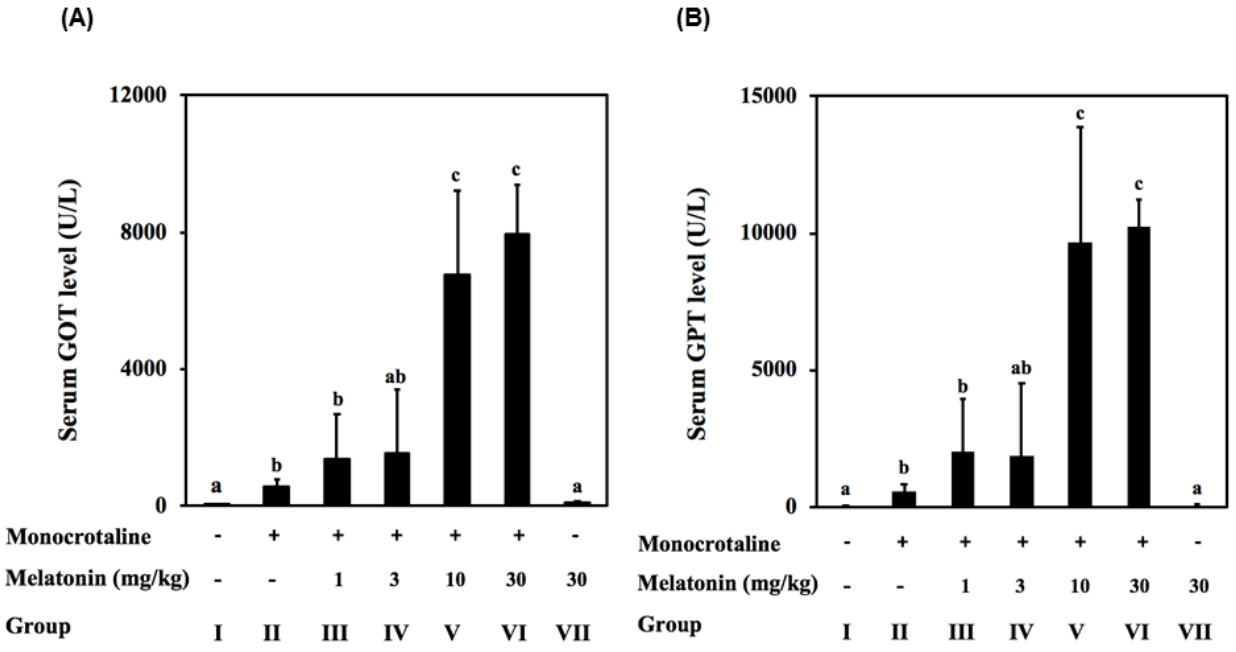


**Figure 1** Effect of melatonin on hepatic dysfunction in SOS. Group I mice were injected with saline. Group II mice were injected with monocrotaline (500 mg/kg). Group III-VI mice were injected with melatonin (1, 3, 10, and 30 mg/kg respectively) and 1 h later injected with monocrotaline (500 mg/kg). Group VII mice were injected with melatonin (30 mg/kg). (A) GOT and (B) GPT levels. All data were expressed as Means ± SD. Data were analyzed by one-way ANOVA followed by Student's *t*-test analysis. [a,b,c]The different letters are statistically significant.

### *3.2 Melatonin Histopathologically Exacerbated SOS*

To examine the histologic role of melatonin in monocrotaline-induced SOS, liver histopathology was assessed. Liver histopathology in melatonin (1-30 mg/kg) treated monocrotaline-induced SOS mice depicted hepatic necrosis, congestion, destruction of sinusoidal structure, and severe hemorrhage in Groups II-VI (Figure 2A). Group II saw a significant increase in histological score (Figure 2B) compared with Group I. Groups III and IV did not show an increase in histological scores compared to Group II. Groups V and VI displayed a significant increase in histological score compared to Group II. There were no alterations in histological score between Group VII compared to Group I.

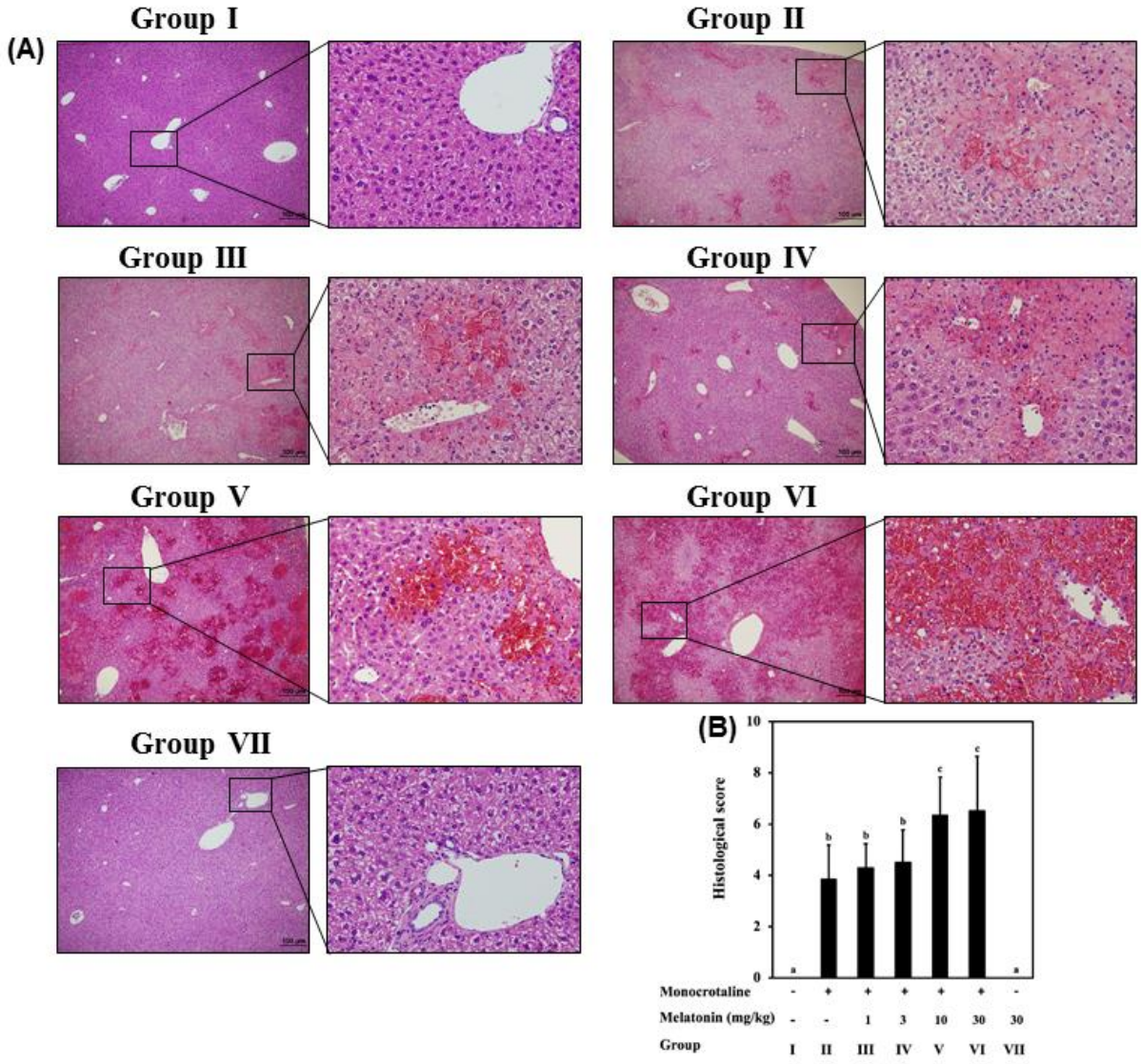


**Figure 2** Effect of melatonin on liver pathology in SOS. For treatment, details refer the Figure 1 legend. (A) Photomicrographs of liver histology (hematoxylin and eosin staining). (B) Histological scoring. Photomicrographs taken at [4 x] × [10 x] and [40 x] × [10 x]. All data were expressed as Means ± SD. Data were analyzed by one-way ANOVA followed by Student's t-test analysis. [a,b,c]The different letters are statistically significant.

### *3.3 Melatonin Decreased Liver Hepatic Collagen*

To study the effect of melatonin on collagen-related hepatic injury in monocrotaline-induced SOS, collagen was assessed histochemically. Masson trichrome staining of liver sections depicted significantly decreased collagen in Groups II-VI (Figure 3A) relative to Group I. The collagen score of Group II significantly decreased (Figure 3B) compared to Group I. In Groups III and IV no alterations were found in the collagen scores compared to Group II. Groups V and VI showed a significant decrease in the collagen score compared to Group II. The mice treated with melatonin-alone in Group VII did not alter the collagen score compared to Group I.

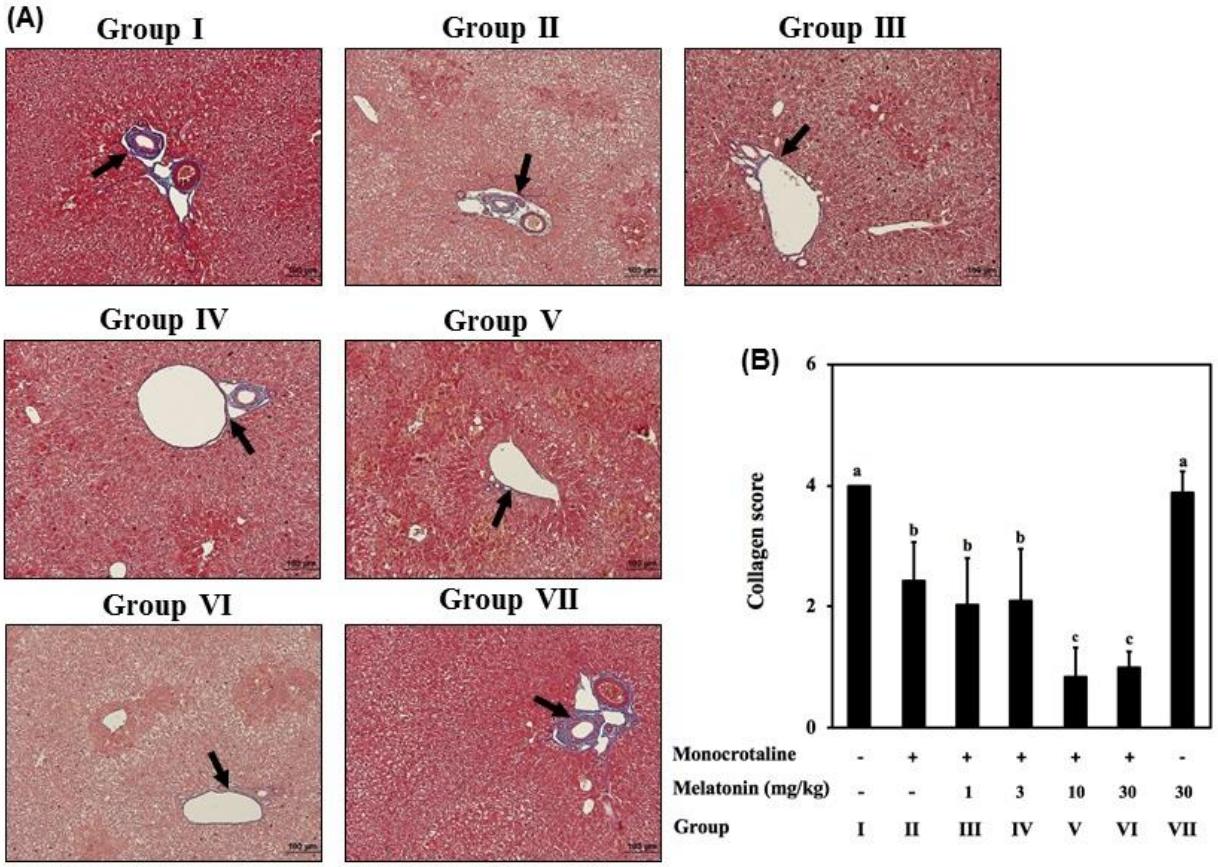


**Figure 3** Effect of melatonin on vessel collagen in SOS. For treatment, details refer the Figure 1 legend. (A) Masson trichrome staining. (B) Collagen scoring. Photomicrographs taken at [10 x] × [10 x]. Arrows show collagen around blood vessels. All data were expressed as Means ± SD. Data were analyzed by one-way ANOVA followed by Student's *t*-test analysis. [a,b,c] The different letters are statistically significant.

### *3.4 Melatonin Decreased RBC, HCT, Lymphocyte, Platelets and Neutrophils in SOS*

To validate the effect of melatonin on blood and immune cells in monocrotaline-induced SOS, the RBC (Figure 4A), HCT (Figure 4B), lymphocytes (Figure 4C), platelets (Figure 4D), and neutrophils (Figure 4E) were analyzed using a haematology analyzer. In Groups II-IV and Group VII animals, no significant alterations were observed in RBC, HCT, and platelets compared to Group I.

However, mice in Groups V and VI showed significantly decreased RBC, HCT, and platelet counts compared to Group II. Lymphocytes were significantly decreased in Group II animals compared to Group I. No change in the lymphocyte count was observed in Groups III or IV, but a significant decrease in Groups V and VI was seen compared to Group II. Neutrophils were significantly increased in Group II animals compared to Group I. Furthermore, Groups III-VI saw significantly increased neutrophil count relative to Group II.

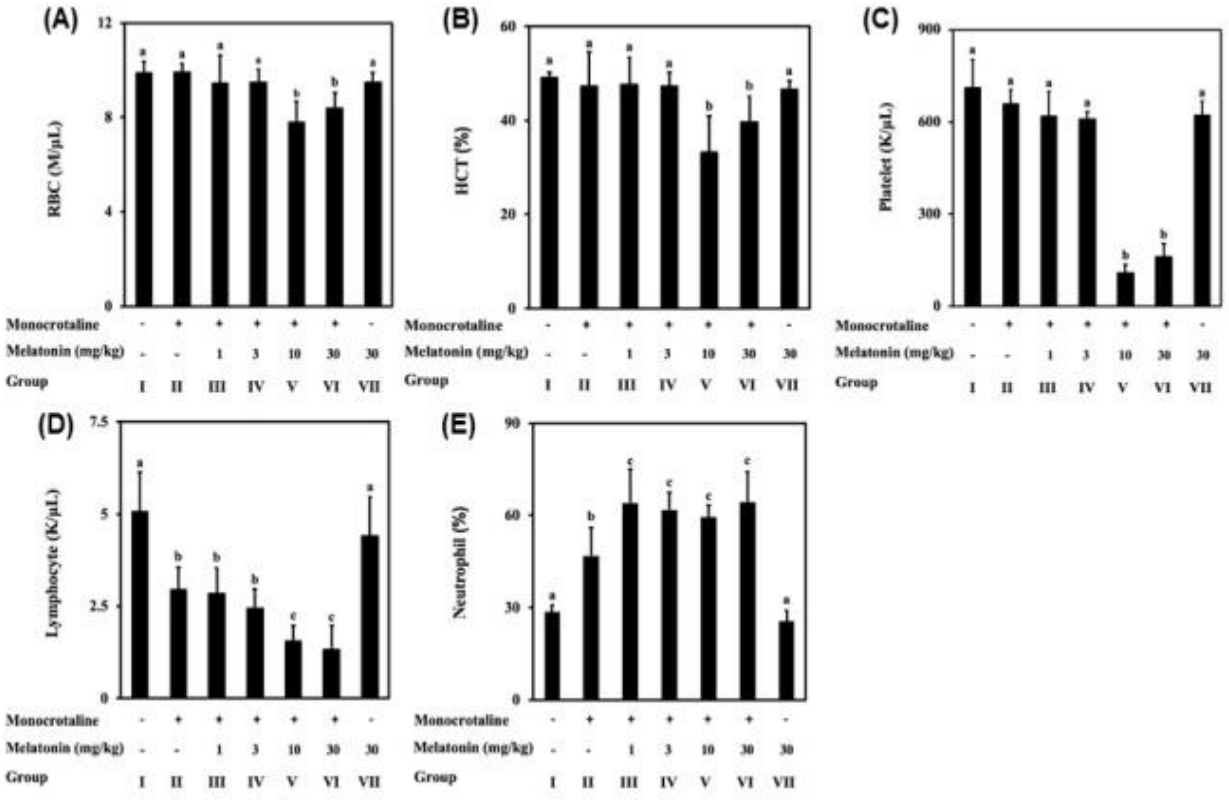


**Figure 4** Effect of melatonin on RBC, HCT, lymphocyte, neutrophils, and platelets in SOS. For treatment, details refer the Figure 1 legend. (A) Red blood cell count; (B) hematocrit ratio; (C) lymphocytes; (D) neutrophils; and (E) platelets. All data were expressed as Means ± SD. Data were analyzed by one-way ANOVA followed by Student's t-test analysis. [a,b,c]The different letters are statistically significant.

### *3.5 Melatonin Upregulated the Expression of CD41 in SOS*

To investigate the role of melatonin in hepatic platelet aggregation in monocrotaline-induced SOS, the expression of CD41 (a marker of platelets) was assessed. A significant increase in the expression of CD41 was noticed in Group II animals (Figure 5) relative to Group I. However, no significant increase in the expression of CD41 was observed in Groups III and IV relative to Group II. In addition, Groups V and VI depicted an increase in the expression of CD41 compared to Group II. Group VII did not show an increase the expression of CD41 compared to Group I.

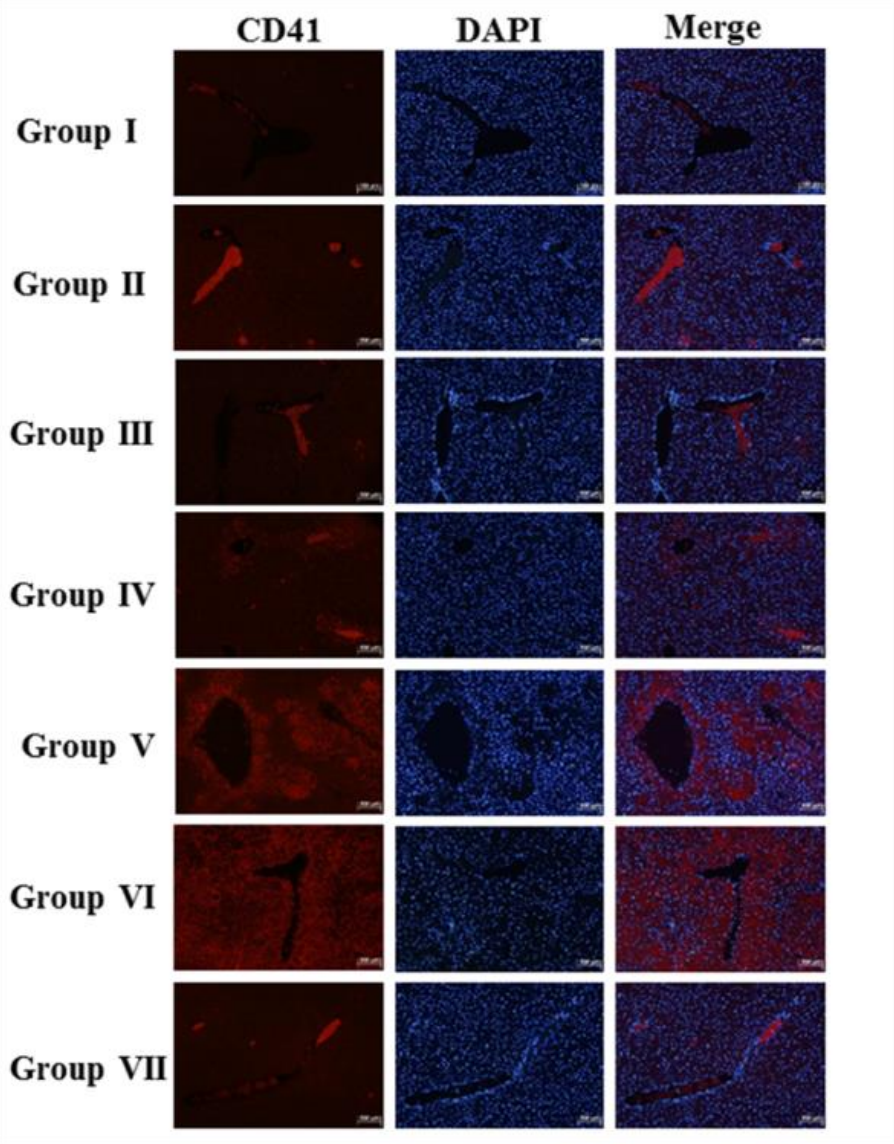


**Figure 5** Effect of melatonin on CD41 immunofluorescence in SOS. For treatment, details refer the Figure 1 legend. Photomicrographs taken at [10 x] × [10 x].

### *3.6 Melatonin Exacerbated the Lipid Peroxidation in SOS*

To examine the effect of melatonin on oxidative stress in monocrotaline-induced SOS, the level of MDA and NO were quantified. The level of MDA (Figure 6A) and NO (Figure 6B) significantly increased in Group II compared to Group I. Melatonin treatment significantly increased MDA and NO in Group III compared to Group II.

### *3.7 Melatonin Upregulated the Expression of NF-κB and iNOS in SOS*

To investigate the role of melatonin on oxidative stress in monocrotaline-induced SOS, the expression of NF-κB and iNOS was assessed. The expression of NF-κB (Figure 6C) and iNOS (Figure 6D) were significantly increased in Group II compared to Group I. Melatonin treatment significantly increased NF-κB and iNOS in Group III compared to Group II. Group IV did not show an increase the expression of NF-κB or iNOS compared with Group I.

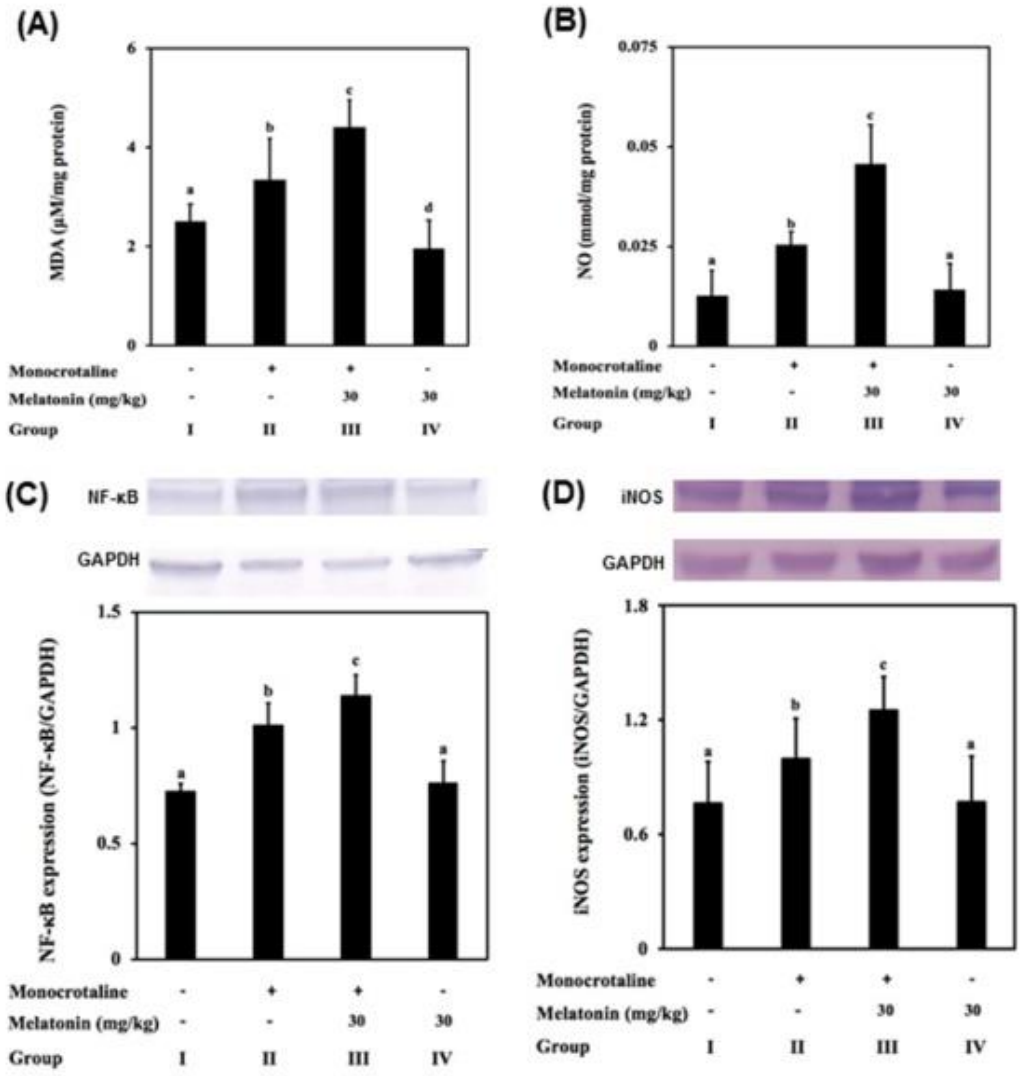


**Figure 6** Effect of melatonin on lipid peroxidation, NF-κB, and iNOS expression in SOS. Group I mice were injected with saline. Group II mice were injected with monocrotaline (500 mg/kg). Group III mice were injected with melatonin (30 mg/kg) and 1 h later injected with monocrotaline (500 mg/kg). Group IV mice were injected with melatonin (30 mg/kg). (A) MDA; (B) NO; (C) NF-κB; and (D) iNOS expression. All data were expressed as Means ± SD. Data were analyzed by one-way ANOVA followed by Student's *t*-test analysis. [a,b,c]The different letters are statistically significant.

### *3.8 Melatonin Decreased Glutathione and Thioredoxin Reductase Level in SOS*

To study the antioxidant effect of melatonin on oxidative stress in monocrotaline-induced SOS, levels of the antioxidants GSH and thioredoxin reductase were assessed. The levels of GSH (Figure 7A) and thioredoxin reductase (Figure 7B) were significantly increased in Group II compared to Group I. In melatonin-treated Group III, there was a significant decrease in the levels of GSH and thioredoxin reductase compared to Group II. In the melatonin-alone treated Group IV, there was a significant increase in the levels of GSH and thioredoxin reductase compared to Group I.

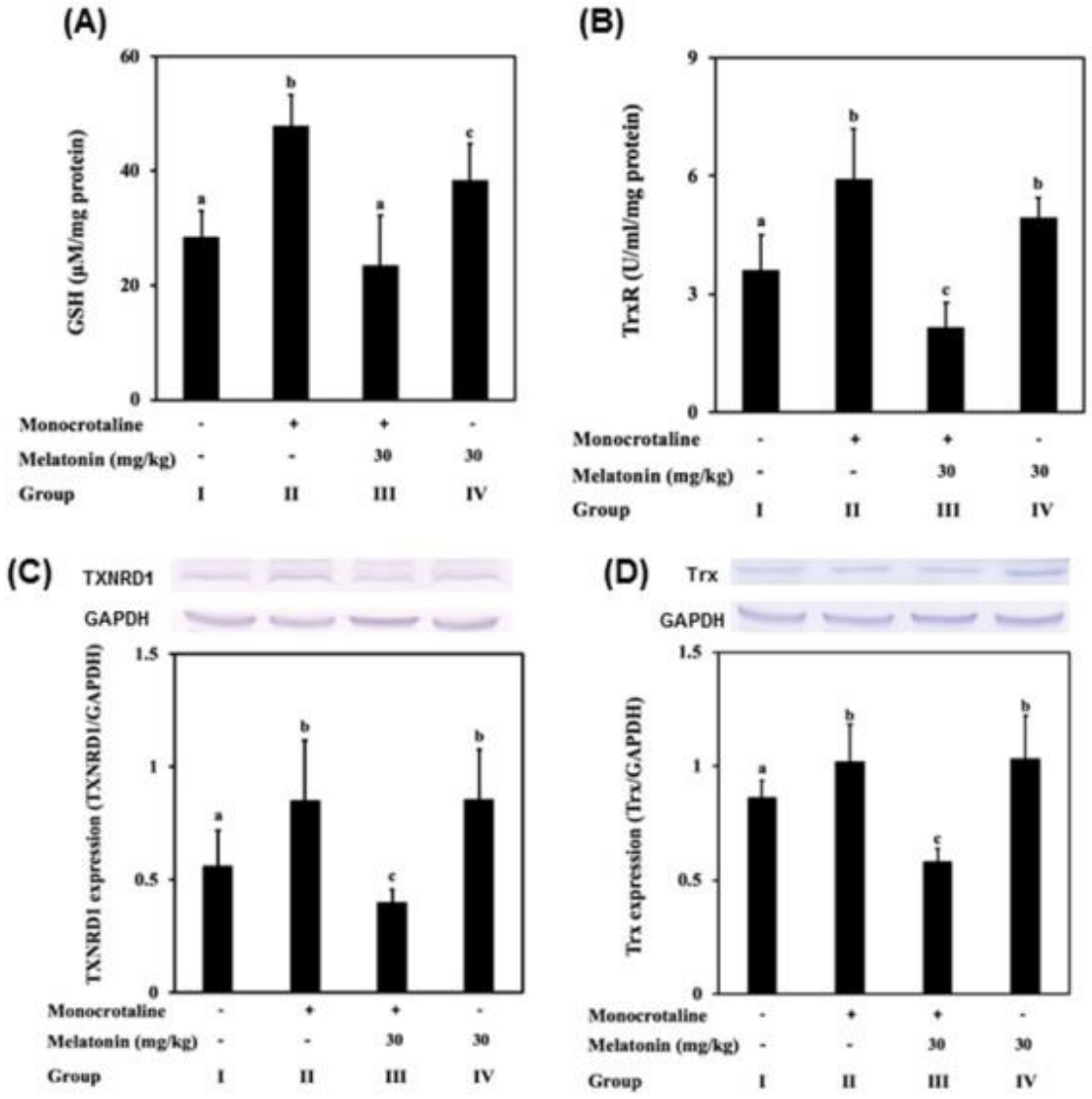


**Figure 7** Effect of melatonin on GSH and TrxR in SOS. For treatment, details refer the Figure 6 legend. (A) GSH; (B) TrxR; (C) TXNRD1; (D) Trx. All data were expressed as Means ± SD. Data were analyzed by one-way ANOVA followed by Student's *t*-test analysis. [a,b,c]The different letters are statistically significant.

### *3.9 Melatonin Downregulated the Expression of TXNRD1 and Trx in SOS*

To investigate the role of melatonin on the expression of TXNRD1 and Trx in monocrotaline-induced SOS, the expressions of TXNRD1 and Trx were assessed. The expressions of TXNRD1 (Figure 7C) and Trx (Figure 7D) in Group II were significantly increased compared to Group I. In melatonin-treated Group III, the expressions of TXNRD1 and Trx were significantly decreased compared to Group II. Melatonin-alone treated Group IV showed significant increases in the expressions of TXNRD1 and Trx compared to Group I. The immunohistochemical expression of Trx (Figure 8A) confirmed the similar expression as a western blot (Figure 8B).

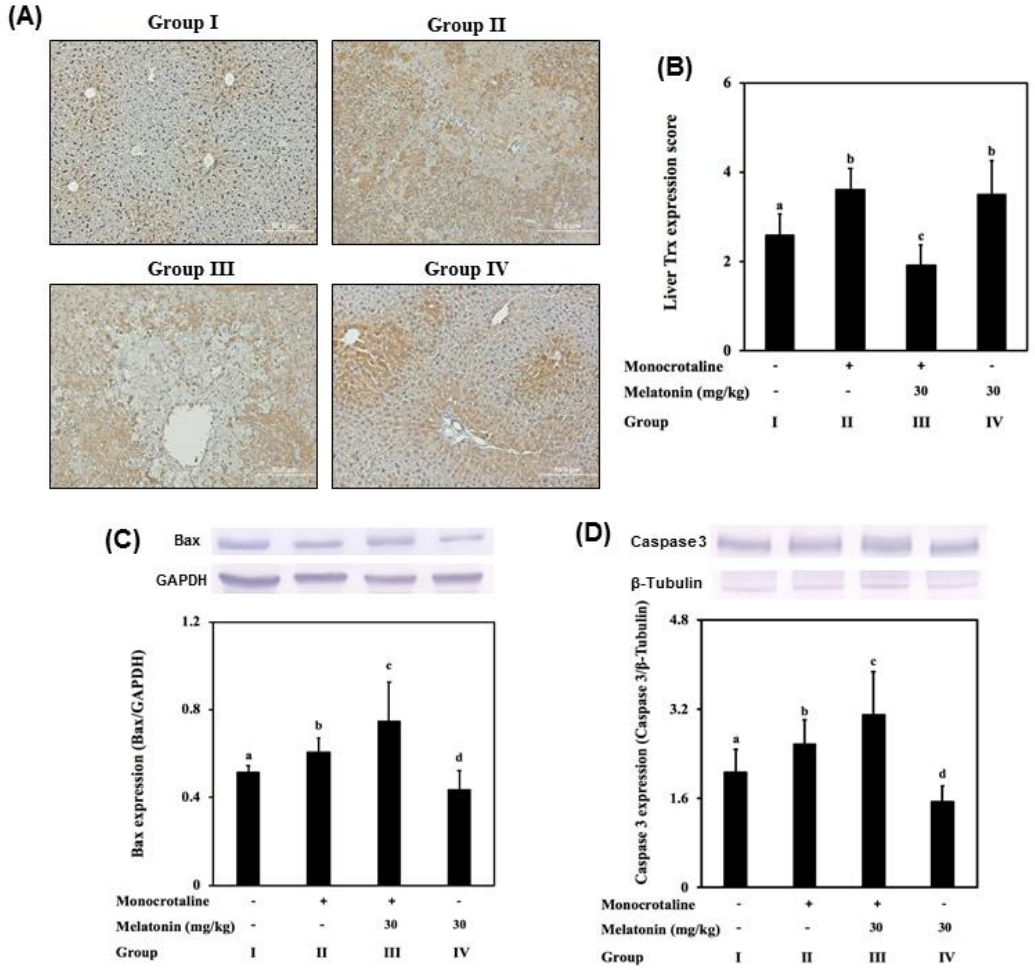


**Figure 8** Effect of melatonin on Trx immunohistochemical expression, Bax, and caspase 3 expression in SOS. For treatment, details refer the Figure 6 legend. (A) Trx immunohistochemical expression; (B) Trx immunohistochemical expression score; (C) Bax; (D) Caspase 3. Photomicrographs taken at [4 x] × [10 x]. All data were expressed as Means ± SD. Data were analyzed by one-way ANOVA followed by Student's *t*-test analysis. [a,b,c,d]The different letters are statistically significant.

### *3.10 Melatonin Upregulated the Expression of Bax and Caspase 3 in SOS*

To elucidate the role of melatonin on apoptosis in monocrotaline-induced SOS, the expression of Bax and caspase 3 was assessed. The expression of Bax (Figure 8C) and caspase 3 (Figure 8D) were significantly increased in Group II animals compared to Group I. In melatonin-treated Group III the expressions of Bax and caspase 3 were significantly increased compared to Group II. In melatonin-alone treated Group IV animals, significant decreases in the expressions of Bax and caspase 3 were seen compared to Group I.

### *3.11 Melatonin Upregulated the Immunofluorescence Expression of ASK1 in SOS*

To study the effect of melatonin on apoptosis in monocrotaline-induced SOS, the expression of ASK1 was assessed. The expression of ASK1 increased in Group II (Figure 9) relative to Group I. In melatonin-treated Group III the expression of ASK1 increased compared to Group II.

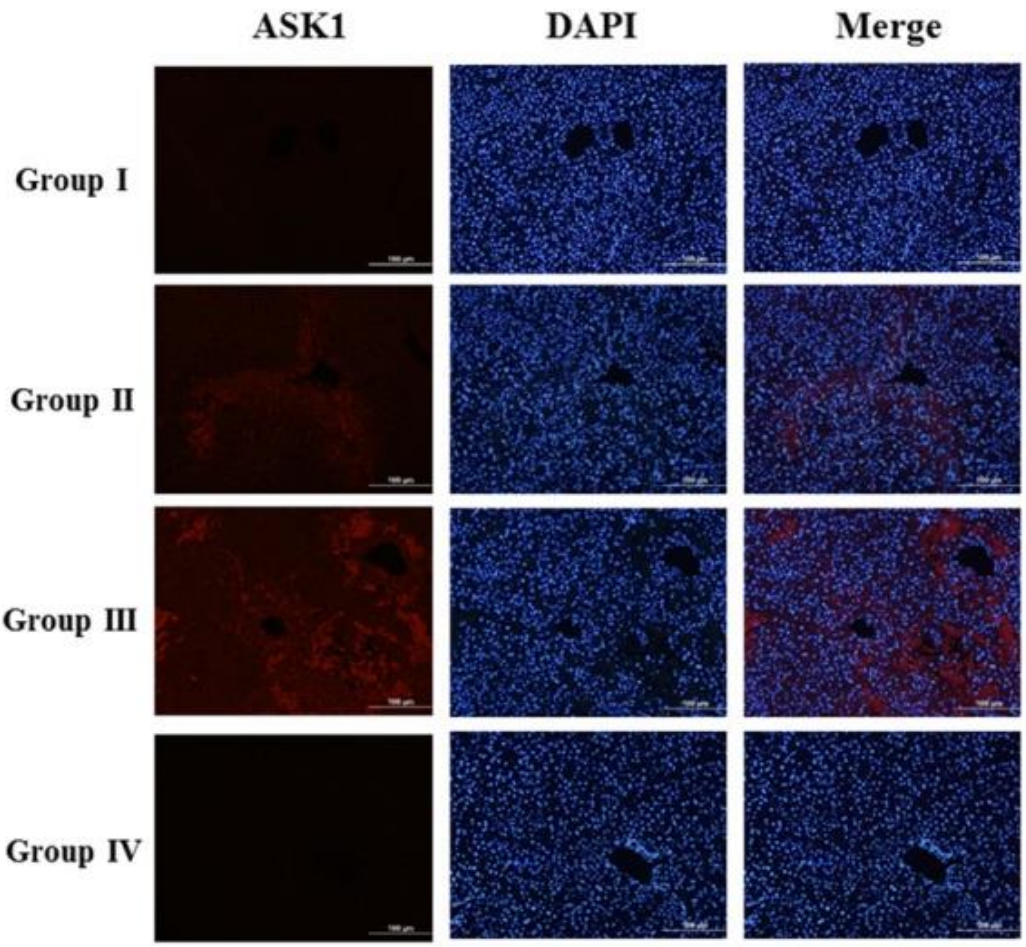


**Figure 9** Effect of melatonin on ASK1 immunofluorescence in SOS. For treatment, details refer the Figure 6 legend. Photomicrographs taken at [10 x] × [10 x].

## 4. Discussion

Melatonin aggravated liver injuries in monocrotaline-induced SOS mice. Liver injury was characterized by elevated serum GOT, GPT, histological score, hepatic platelet aggregation, and neutrophil count. In addition, melatonin decreased hepatic collagen, RBC, HCT, platelets and lymphocytes. Furthermore, melatonin activated oxidative stress by elevating liver MDA and NO levels and quenching antioxidants GSH and TrxR, ultimately leading to activation of apoptosis signalling pathways such as ASK1, Bax, and caspase 3 in monocrotaline-induced SOS.

In this study, melatonin exacerbated liver injuries and SOS. In the process of liver injury, the hepatic cells leaked GOT and GPT enzymes into the blood, thereby elevating circulating GOT and GPT. Melatonin treatment elevated the serum GOT and GPT levels in MCT-induced SOS, indicating an aggravation of the exacerbated hepatic injury. Hepatic hemorrhage is a pivotal sign of SOS [22, 25]. The metabolite of monocrotaline degrades the basement membrane collagen of sinusoids, leading to obstruction of blood in the sinusoids. In the present study, red blood cell congestion and hemorrhage in between hepatocytes were depicted in SOS. Furthermore, melatonin accelerated the breakdown and loss of collagen in SOS. The breakdown of collagen in blood vessels leads to damage of central and portal veins, ultimately causing SOS that increases cell membrane damage and apoptosis of hepatocytes [20, 21].

Melatonin decreased the blood platelet count, RBC, and HCT in monocrotaline-induced SOS. Reduction in platelets, RBC, and HCT is found in hepatic impairment, anaemia, acute hemorrhage, and hemolysis [26]. In addition, monocrotaline-induced SOS is characterized by hepatic hemorrhage [27]. In the present study, melatonin, a powerful antioxidant, was used to attenuate SOS. However, melatonin reduced the platelet count, thereby altering blood coagulation hemostasis, leading to elevated hepatic hemorrhage [13]. In response to injury to hepatic sinusoids, platelets migrate to the injured site to aid in healing the injury, mainly in the central and portal veins, leading to platelet aggregation (thrombosis). This obstructs the flow of blood through the circulatory system, causing accelerated liver injury in SOS [28, 29]. Melatonin affects blood coagulation hemostasis in monocrotaline-induced SOS; therefore it decreases the blood platelet count, RBC, and HCT, leading to severe liver injury with considerable hemorrhage.

Melatonin reduced the lymphocyte and elevated the neutrophil counts in monocrotaline-induced SOS. The neutrophil is the major innate immune system that protects against liver injury. During liver injury, the homeostasis of neutrophils are altered. Neutrophil elevation was observed in acute poisoning, acute hemorrhage, and/or hemolysis. Neutrophils were recruited in monocrotaline-induced SOS [30]. Melatonin elevated monocrotaline-induced neutrophil recruitment in SOS. The reduction in the number of lymphocytes may be due to acute hemorrhage. During severe liver injury, lymphocytes are produced to compensate for the injury; lack of lymphocyte activity may ultimately lead to the destruction of liver cells. In the present study, monocrotaline reduced blood lymphocytes. Furthermore, the neutrophil to lymphocyte ratio (NLR), which is calculated by dividing the number of neutrophils by number of lymphocytes, has been a novel index and effective marker associated with various inflammatory and oxidative stress issues [31]. A high NLR value indicates a high risk of acute injury. Melatonin administration depicted a high NLR value indicating acute SOS.

Melatonin elevated oxidative stress and quenched antioxidants in monocrotaline-induced SOS. ROS has stimulatory roles in NF-κB signalling. Monocrotaline upregulated the expression of NF-κB and increased the hepatic LPO product MDA level. Furthermore, monocrotaline increased liver NO [32, 33]. Both NF-κB activation and iNOS expression lead to elevated oxidative damage. Monocrotaline administration decreased GSH because the experiment durations were 48 h to 96 h; therefore, oxidative stress overpowers GSH oxidation [34]. In the present study, the experimental duration was 24 h; the increase in GSH might be due to initial protective mechanisms that counteract monocrotaline-induced oxidative stress. The liver releases a lot of GSH to counteract hepatic damage. An imbalance exists between antioxidants and ROS in the liver, which is considered crucial in the development of oxidative stress and exacerbating liver damage [27]. The interaction between melatonin and monocrotaline caused severe liver injury, resulting in the oxidation of GSH. Melatonin elevated MDA and NO levels and oxidized GSH in monocrotaline-induced SOS. In the present study, we injected melatonin one hour prior to monocrotaline administration. Monocrotaline was found to initially cause hepatic hemorrhage, slowly building up to severe hemorrhage, particularly in the central and portal veins of the liver leading to platelet aggregation, known asthrombosis. This entire process of hepatic hemorrhage, platelet aggregation, and thrombosis is characterized by elevated oxidative stress. In addition to this, injected melatonin was found to decrease blood coagulation.Blood thus enters the injured liver tissue causing severe hemorrhage leading to accelerated liver injury in SOS. SOS is characterized by an

increase in hemorrhage and oxidative stress. Melatonin was found to increase hemorrhage, thereby elevating SOS, ultimately increasing the oxidative stress.

Melatonin downregulated the expressions of Trx and TXNRD1 in monocrotaline-induced SOS. Monocrotaline and melatonin were found to have a common binding protein, TXNRD1 for its action, which is found in the cytosol and plays a critical role in the regulation of endothelial intracellular proteins and is associated with regulation of hepatic TrxR activity [35, 36]. TrxR controls the redox state of cysteine residues in proteins and has numerous roles in redox regulation. TrxR reduces lipid hydroperoxides and hydrogen peroxide by transferring electrons from NADPH to Trx, which in turn reduces Trx peroxidase. In the present study, monocrotaline and melatonin individually upregulated the expression of Trx and TXNRD1. However, melatonin downregulated the expression of Trx and TXNRD1 in monocrotaline-induced SOS. In this study, we administered melatonin 1 h before monocrotaline treatment. Monocrotaline and melatonin are known to have a common binding protein, Trx [36, 37]. Therefore, the interaction between monocrotaline and melatonin caused severe liver damage in mice, resulting in downregulated expression of Trx and TXNRD1, which reduced hepatic TrxR level. This leads to insufficient antioxidant activity against oxidative stress, which leads to activation of apoptosis pathways [38, 39]. In addition, inhibition of Trx and TXNRD1 expressions directly lead to the activation of ASK1 expression [40]. ASK1 is a ROS-sensitive mitogen-activated protein kinase. Oxidative stress leads to overexpression of ASK1 which induces apoptotic cell death. Activated ASK1 induces the activation of JKN/p38 downstream signalling pathways, and leads to apoptosis by the caspase 3 pathway [41]. Activation of JKN/p38 induces the release of the proapoptotic factor Bax [42]. Bax activation results in its translocation to mitochondrial disruption, which leads to apoptosis [43]. Melatonin did not attenuate monocrotaline-induced SOS; on the contrary, melatonin aggravated liver injury through TXNRD1/Trx/ASK1/Bax/Caspase 3 apoptosis signalling pathways (Figure 10).

Melatonin exerts a protective role against oxidative stress; however, the indoleamine can also induce a pro-oxidant environment, which has been related to a cytotoxic effect. The interest in the role of melatonin in the regulation of cellular physiology is widespread. Many cell types express membrane-bound melatonin receptors, which include melatonin type 1 (MT1) and melatonin type 2 (MT2) receptors. Their expression differs among various organs. Additionally, melatonin can bind to cytosolic and nuclear orphan receptors from the RORα/RZR family. The indole regulates enzyme and bicarbonate secretion. Although the vast majority of studies prove the antioxidant capacity of melatonin and its derivatives, a few studies using cultured cells found that melatonin promoted the generation of ROS at pharmacological concentrations in different cells. Thus, melatonin functioned as a conditional pro-oxidant. This action may highly depend on the concentration of melatonin used. The GSH/GSSG ratio changed in the presence of melatonin in a concentration-dependent manner. The drop in the GSH/GSSG ratio that reflects an increase in the oxidized form glutathione might be related to a pro-oxidant effect of melatonin, which is augmented with an increasing concentration of the indole. Additionally, the indole induced a concentration-related generation of ROS, which was detected both in the mitochondria and in the cytosol. Conversely, melatonin induced significant increases in ROS production. These results could be related to the decrease in the GSH/GSSG ratio; i.e. a higher concentration of melatonin leads to a higher oxidation state [44].

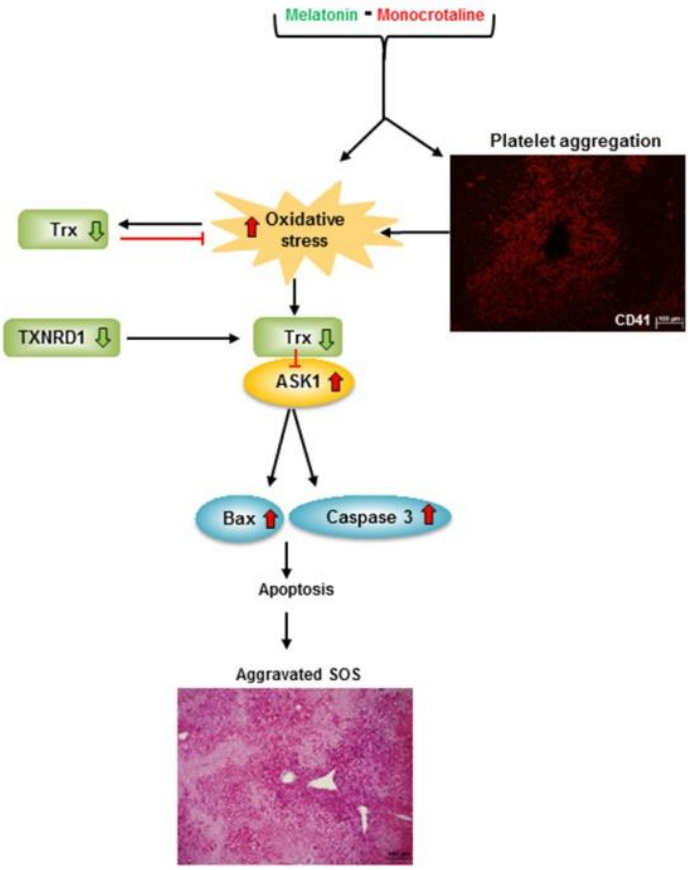


**Figure 10** Possible mechanism of melatonin aggravation of monocrotaline-induced sinusoidal obstruction syndrome. SOS: Sinusoidal obstruction syndrome; TXNRD1: Thioredoxin reductase 1; Trx: Thioredoxin; ASK1: Apoptosis signal-regulating kinase 1; Bax: Bcl-2-associated X protein.

The clinical implication of the study is that melatonin benefits patients undergoing chemotherapy, radiotherapy, supportive therapy, and/or palliative therapy by improving survival and attenuating the adverse effects of chemotherapy. In addition, it is prescribed for a variety of conditions during chemotherapy, such as for use as an antioxidant supplement, adjutant, antiproliferative, immune modulator and hormone modulator, and also to aid in chemotherapy-related insomnia [45]. Under clinical treatment, chemotherapy-induced SOS is treated using various chemicals and monoclonal antibody conjugates such as defibrotide, anti-thrombotic, thrombolytic agents, calicheamicin, gemtuzumab, and inotuzumab. However, these agents may cause adverse effects, including fatal hamorrhage, mild fever, hypotension, liver enzyme elevations, epigastric pain, weight gain, peripheral neuropathy, and insomnia [19]. These adverse effects pose a life threat to cancer patients. Our primary aim of the experiment was to treat SOS without adverse side effects, using a well-known antioxidant and sleep medicine, melatonin. However, the results of the study were not promising, as melatonin actually aggravated SOS in our experiment. Thus, the current study provides a potential warning for cancer patients undergoing melatonin treatment as an antioxidant supplement, adjutant, antiproliferative, immune and/or hormone modulator, or to combat chemotherapy-related insomnia during oxaliplatin-based chemotherapy, with the ultimate aim of preventing severe hepatic injury/failure.

## 5. Conclusions

Melatonin may aggravate oxaliplatin-mimicking sinusoidal obstruction syndrome via hepatic platelet aggregation and oxidative stress, ultimately leading to activation of apoptosis.

## Author Contributions

Periasamy S. conceived of the presented idea. Chien S.-P. and Li B.-F. designed and performed the experiments, derived the models and analysed the data. Periasamy S. and Hsu D.-Z. wrote the manuscript in consultation with Liu M.-Y. All authors discussed the results and contributed to the final manuscript.

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## Competing Interests

The authors have declared that no competing interests exist.

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