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Research Article

Melatonin Protects Against Cyclophosphamide-Induced Hepatic and Renal Alterations in Rats.

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ABSTRACT

Cyclophosphamide (CLP), a cytotoxic alkylating agent with immunosuppressive and antitumor properties is used in the treatment of different types of cancers, but it is known to cause toxicity-induced changes to the body tissues. Melatonin, an antioxidant mainly secreted by the pineal gland has protective properties especially against tissue toxicity. This study was aimed at investigating the role of melatonin (MLT) in cyclophosphamide-induced toxicity of the liver and kidney using serum biochemical analysis and histopathology in adult Wistar rats. Twenty-four adult male Wistar rats were grouped into four (n=6): group 1 was injected intraperitoneally with 0.2mL of normal saline for 14 days, group 2 was injected with 10mg/kg of melatonin intraperitoneally for 14 days, group 3 was injected intraperitoneally with 0.2mL of normal saline for 14 days and 150mg/kg of CLP on the 15th day and in group 4, the rats were injected with 10mg/kg of melatonin for 14 days and 150mg/kg of CLP on the 15th day. Forty-eight hours after the last treatment, the rats were weighed; blood samples collected for biochemical analysis while liver and kidney samples were processed for histology. The results revealed that CLP-treated rats had hypokalemia and hypochloremia with a significant increase in the levels of liver and kidney function markers. Histopathological analysis showed congested central vein and widened sinusoids in the liver, while there were widened as well as congested urinary spaces and loop of Henle, with loss of glomerular epithelia in the kidneys. The rats treated with melatonin and CLP showed improvement in body weight, biochemical parameters of hepatic and renal functions as well as improved tissue conditions. In conclusion, a pre-treatment with melatonin is recommended in cyclophosphamide therapy.

Keywords: *Cyclophosphamide, melatonin, liver and kidney toxicity.*

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INTRODUCTION

Cancer treatment as well as management is currently witnessing a number of aggressive chemotherapy and radiotherapy; however, chemotherapy for cancer is not specific for tissues, hence both normal and cancerous tissues are being damaged (Christina, 2008; Abdel-Hafez *et al*., 2017). Cyclophosphamide (CLP), a cytotoxic alkylating bi-functional synthetic agent belonging to nitrogen mustard class, is a common anticancer or anti-tumour chemotherapeutic agent, which works mainly by suppressing the immune system. CLP is commonly used in the treatment of lymphoma, multiple myeloma, leukaemia, ovarian cancer, breast cancer, small cell lung cancer, neuroblastoma and sarcoma. CLP has been demonstrated to be carcinogenic thereby increasing the possibilities of developing a number of cancers including lymphomas, leukaemia, skin cancer, transitional cell carcinoma of the bladder or other malignancies (Bernatsky and Clarke, 2008; Sakr *et al*., 2017). Cyclophosphamide toxicity has been reported for the liver (Li *et al*., 2010), kidney (Sakr *et al*., 2017) and testis (Turner and Lysiak, 2008; Turk *et al*., 2010). Some studies have shown that intraperitoneal administration of cyclophosphamide in rats could result in severe tissue oxidative stress (Bhatia *et al*.,

2006; Abdel-Hafez *et al*., 2017), and massive cellular damage (Hanaa *et al*., 2010), triggering apoptosis (Napolitano and Singh, 2002), which leads to the eventual death of normal and cancerous cells (Stankiewicz *et al*., 2002; Abdel-Hafez *et al*., 2017). Melatonin (N-acetyl-5-methoxytryptamine), secreted by the pineal gland in humans and animals, is the hormone responsible for the regulation of sleep and wakefulness (Olukole *et al*., 2018). Melatonin (MLT) is also secreted by some other tissues apart from the pineal gland, including the liver, skin and small intestine (Hardeland *et al*., 1995; Rocha *et al*., 2015). In mammals, the highest circulating levels of MLT in blood has been demonstrated during sleep, especially that a number of sleep and mood disorders including jet lag and shift workers' conditions have been traced to disequilibrium of MLT secretion and/or production (Lam *et al*., 1990; Arendt *et al*., 1997; Rocha *et al*., 2015). MLT is also produced in plants, functions as a first line of defence against oxidative stress (Hardeland, 2005).

Several therapeutic agents and or antioxidants have been used to ameliorate the damaging effects of cyclophosphamide. Sakr *et al*. (2017) demonstrated the ameliorative effect of fennel oil in cyclophosphamide-induced toxicity of the kidney of rats. El-Naggar *et al*. (2015) reported the ameliorative effects of propolis against cyclophosphamide-induced toxicity of the kidney and liver of mice. The ameliorative effect of royal jelly on cyclophosphamide-induced prostatic damage in male rats has also been reported (Abdel-Hafez *et al*., 2017). With the exception of the report of Shokrzadeh *et al*. (2014) where graded doses of MLT were used during a seven-day duration to ameliorate cyclophosphamide-induced toxicity in the liver of mice, there is the paucity of research information on the role of MLT in cyclophosphamide-induced toxicity in rodents. This study was therefore designed to investigate the role of MLT in cyclophosphamide-induced toxicity of the liver and kidney of adult Wistar rats.

MATERIALS AND METHODS

Chemicals: CLP as well as MLT used for the study were purchased from Sigma-Aldrich Co, St Louis, Missouri, United States of America. All other reagents used in the study were of standard grade.

Experimental Animals: All procedures used in the study were carried out according to the National Institutes of Health's protocol on handling of laboratory animals for biomedical research (Garber *et al*. 2011). Twenty-four male Wistar rats (average weight of 190 g) obtained from the Experimental Animal Unit, Faculty of Veterinary Medicine, University of Ibadan, Nigeria, were used for the study. The rats were kept in plastic cages under controlled environmental conditions, being kept on commercial rat pellets and clean water provided *ad libitum*. The rats used in the study were divided into four groups (n=6):

- Group I (Control): Rats received 0.2 mL normal saline intra-peritoneally for 14 days.
- Group II (MLT): Rats received intra-peritoneal 10 mg/kg/day MLT for 14 days.
- Group III (CLP): Rats received normal saline intra-peritoneally for 14 days followed by a single dose of CLP on the 15th day in a dose of 150 mg/kg intra-peritoneally.
- Group IV (MLT + CLP): Rats received intraperitoneal 10 mg/kg/day MLT for 14 days followed by a single dose of CLP 150 mg/kg/day intraperitoneal at the 15th day.

The dosage, duration and route of administration of MLT and CLP used in the study were as reported by Olukole *et al.* (2018), Abdel-Hafez *et al*. (2017), respectively.

Sample Collection: 48-hours post-CLP administration, rats were weighed, anaesthetized under light diethyl ether; blood samples collected in plain tubes from each rat and centrifuged (centrifuge Jantezki, T30, Germany), at 5000 rpm for 10 min for serum collection. Sera collected were separated and refrigerated at -20 °C until analysis. Liver and kidney samples were harvested and fixed in buffered neutral formalin and processed for histological analysis.

Serum Biochemical Analysis: Serum Na^+, K^+, Cl^- and HCO_3^- were determined by use of automated analyzers (Meyer and Harvey, 1988). Commercially available kits were used according to the respective manufacturer's protocol for the measurement of serum liver function enzyme activity. As described by Ola-Davies *et al*. (2014), Serum alkaline phosphatase (ALP) activity was determined by a kit from BioSystems SA., Spain. Serum aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), alanine transaminase (ALT), acid phosphatase and prostatic acid phosphatase activities, Urea, Creatinine, Total Bilirubin (TB), Conjugated Bilirubin (CB), Total Protein (TP) were measured using RANDOX® laboratory reagent kits obtained from RANDOX Laboratories Ltd., Ardmore, United Kingdom.

Histopathology: Liver and kidney samples were collected in 10% neutral buffered formalin for histological analysis. Tissues were processed and embedded in paraffin wax and sections were made of about 4-6 µm. After staining with haematoxylin and eosin, slides were examined under the microscope (Olympus, Japan) for histopathological changes and photographed.

Statistical Analysis

Data were expressed as means ± SD. Means were compared using One-Way ANOVA. Statistical significance was considered at $p< 0.05$. Presentations of data in graphical formats were carried out with the aid of GraphPad Prism 5 software (La Jolla, California, USA).

RESULTS

The effect of MLT on CLP-induced alterations in body, liver and kidney weights are given in figure 1 A-C. There was no significant decrease in body weight of CLP-treated rats compared to the control (figure 1A). Similarly, CLP induced reduction in kidney and liver weights compared to the control rats although the differences were not significant (figure 1B & C). Also, the pre-administration of MLT with CLP resulted in none significant increases in body as well as organ weights across the groups (figure 1A-C).

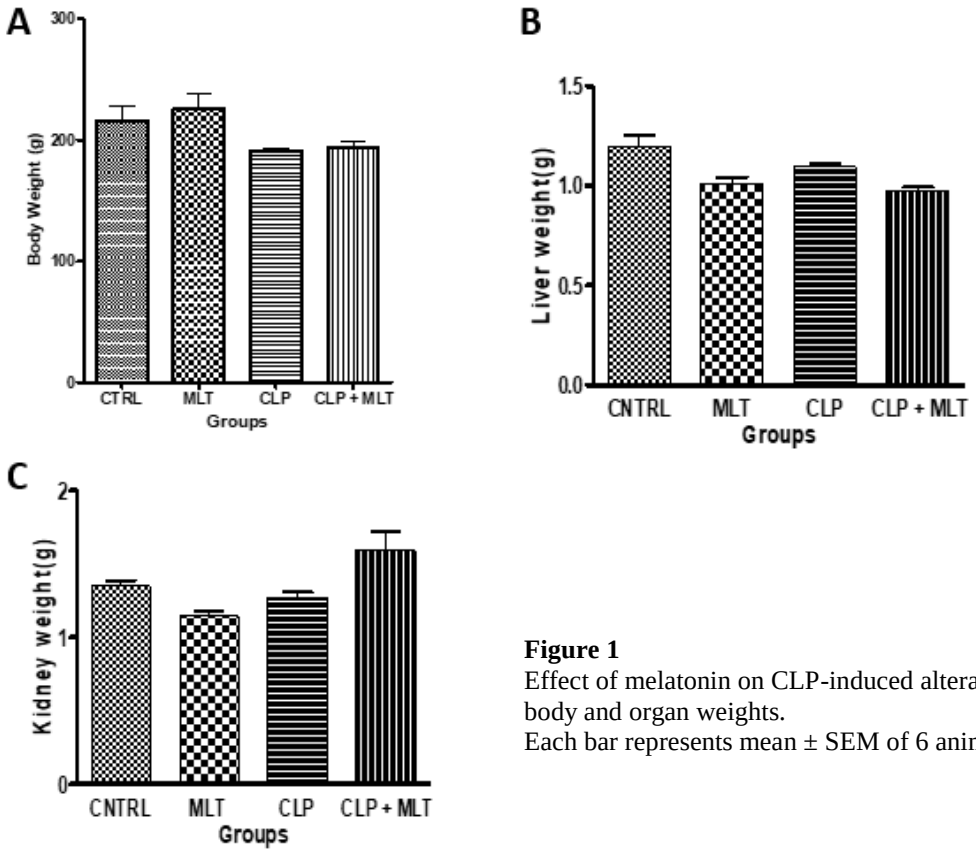

Figure 1
Effect of melatonin on CLP-induced alterations in body and organ weights.
Each bar represents mean ± SEM of 6 animals

Table 1:
Effect of melatonin on cyclophosphamide-induced changes in serum electrolytes of rats.

Electrolytes	Control	MLT	CLP	MLT+CLP
Na^+	138.3 ±1.86[a]	140.0 ±0.89[a]	112 ±0.12[b]	140.0 ±0.89[a]
K^+	4.43 ±0.05 [a]	3.93 ±0.14 [a]	2.07 ±0.14 [b]	4.03 ±0.05 [a]
Cl^-	108.3 ±2.58 [a]	105.0 ±4.47 [a]	98 ±2.58 [b]	108.0 ±2.58 [a]
HCO_3^-	24.33 ±0.52	23.00 ±1.79	24.00 ±1.79	21.00 ±1.86

Means with different superscripts within rows are significantly different (P<0.05)

Table 2:
Effects of melatonin on cyclophosphamide-induced changes in serum proteins of rats

Serum Proteins	Control	MLT	CLP	MLT+CLP
Albumin	3.97±0.05[a]	3.90±0.09[a]	3.07±0.26 [b]	4.00±0.15[a]
Globulin	3.03±0.05[a]	3.13±0.10[a]	2.17±0.10 [b]	3.03±0.05[a]
Total Protein	7.00±0.09[a]	7.03±0.18[a]	5.73±0.23 [b]	7.07±0.21[a]

Means with different superscripts within rows are significantly different (P<0.05)

The effects of MLT on the electrolytes of CLP-treated rats are given in Table 1. CLP significantly reduced the levels of Na^+, K^+ and Cl^- compared to the control while the pre-treatment with MLT increased the levels of Na^+, K^+ and Cl^-. However, there were no significant differences between the control and CLP groups for HCO_3^-. Cyclophosphamide induced significant decrease in the levels of serum proteins (albumin, globulin and total protein) compared to the control while the pre-treatment with MLT protected against these alterations (table 2).

With respect to liver function parameters, there were significant increases ($p<0.05$) in the levels of ALP, ALT, AST and GGT in the CLP-treated rats compared to the control (figure 2A-E).

Conversely, CLP did not induce any significant difference in the levels of CB compared to the control (figure 2E). However, the pre-treatment with MLT protected against the CLP-induced increases in ALP, ALT, AST and GGT levels. Kidney function tests revealed significant differences between the CLP-treated rats and the control for both serum creatinine and urea (Figure 3A-B).

The pre-treatment with MLT attenuated the observed elevations in serum creatinine and urea. Histologically, the control and MLT-treated rats revealed normal mono-nucleate and bi-nucleate hepatocytes in the liver parenchyma with normal central veins and sinusoids (Plate 1A and B). However, the CLP-treated rats had congested central veins and widened sinusoids (Plate 1C-E). The pre-administration of MLT with CLP attenuated these alterations in liver architecture (Plate 1F).

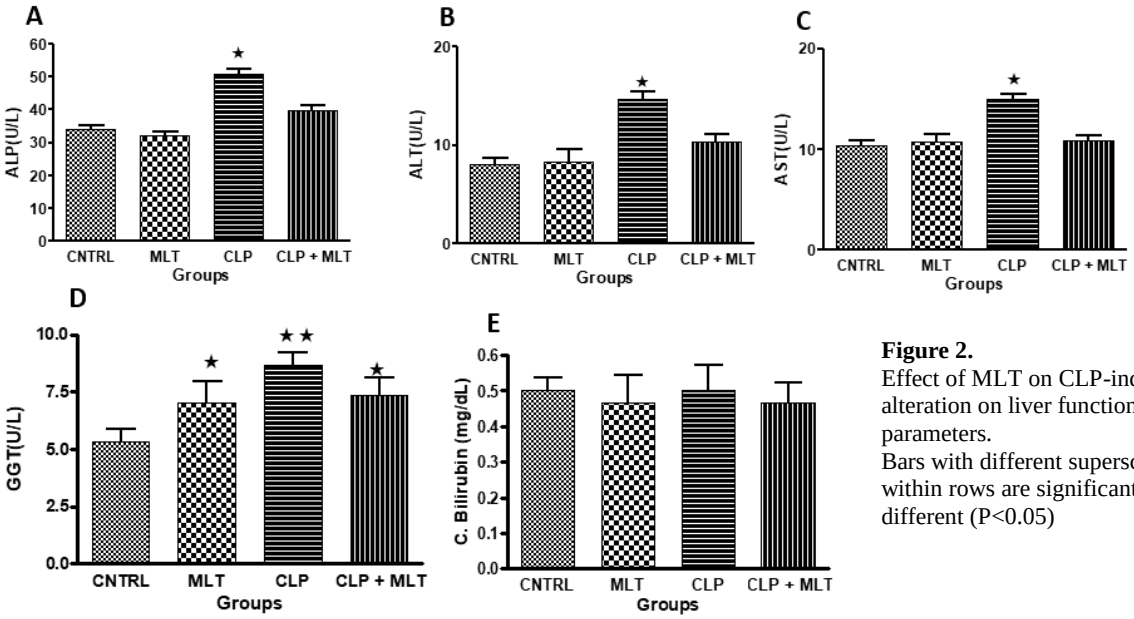

Figure 2.
Effect of MLT on CLP-induced alteration on liver function parameters.
Bars with different superscripts within rows are significantly different (P<0.05)

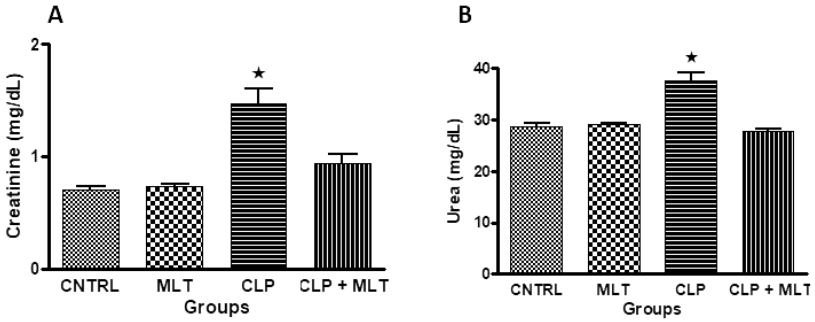

Figure 3:
Effect of melatonin on cyclophosphamide-induced alterations on kidney function parameters.
Bars with different superscripts within rows are significantly different (P<0.05).

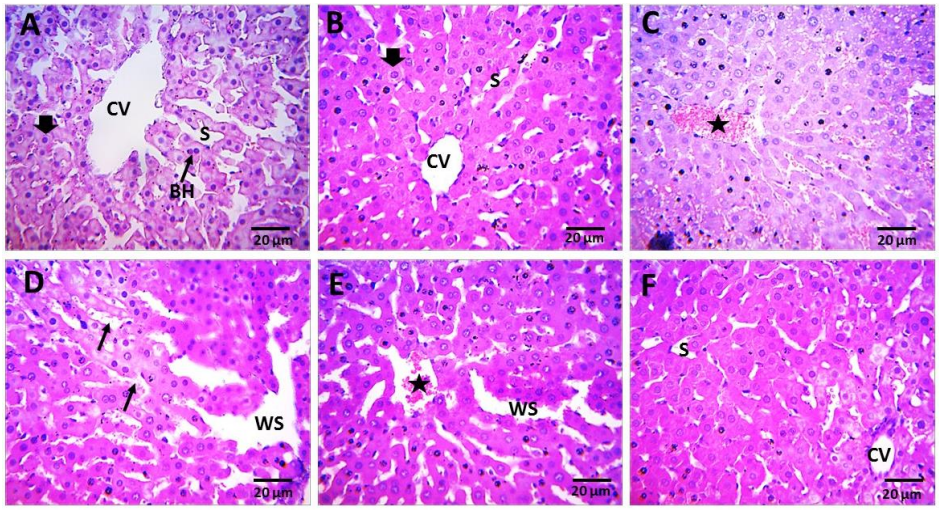

Plate 1.
Photomicrographs showing the effect of melatonin on cyclophosphamide-induced changes in the liver of adult male rats
*A. Control rats showing normal central vein **(CV)**, normal sinusoid**(S)**, mono-nucleate **(block arrow)** and bi-nucleate **(arrow)** hepatocytes.*
*B. MLT treated rats showing normal hepatic plate, normal central vein **(CV)**, normal sinusoid **(S)** and mono-nucleate hepatocyte **(block arrow)**.*
*C. CLP treated rats showing congested central vein **(star)**.*
*D. CLP treated rat showing widened sinusoids **(WS)**.*
*E. CLP treated rat showing congested central vein **(star)** and widened sinusoid **(WS)**.*
*F. MLT and CLP treated rat showing restored hepatic plate with normal sinusoid **(S)** and normal central vein **(CV)**.*

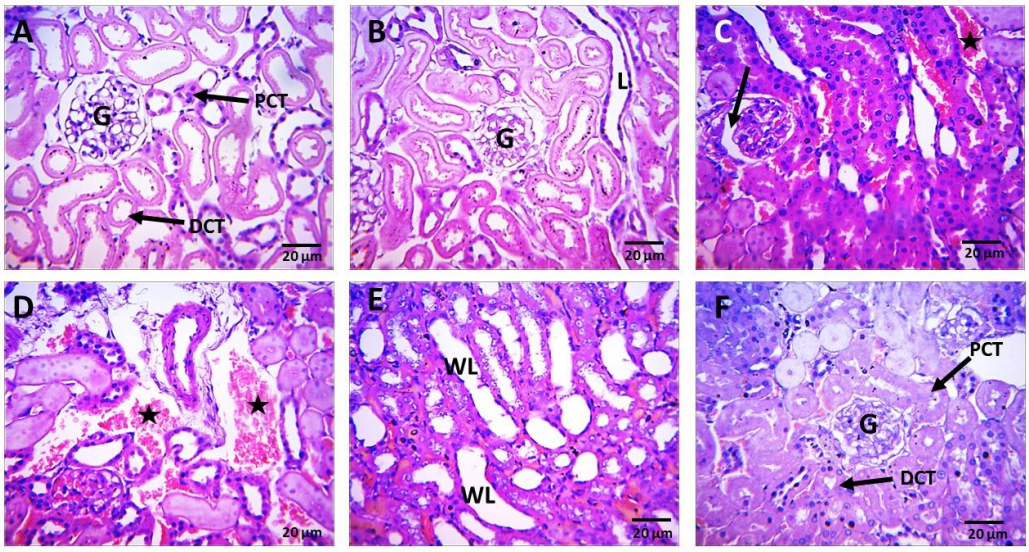

Plate 2.
Photomicrographs showing the effect of melatonin on cyclophosphamide-induced changes in kidneys of adult male rats.

*A. Control group of rats showing normal glomerulus (**G**), normal proximal convoluted tubule (**PCT**), normal distal convoluted tubule (**DCT**).*
*B. MLT treated rats showing normal renal corpuscles, normal glomerulus (**G**), normal loop of Henle (**L**).*
*C. CLP treated rats showing congested loop of Henle (**star**), loss of glomerular epithelia and widened urinary space (**arrow**).*
*D. CLP treated rats showing congested urinary spaces (**star**)*
*E. CLP treated rats showing widened loop of Henle (**WL**).*
*F. MLT and CLP treated rats showing restored renal corpuscles, glomerular epithelium (**G**), restored proximal convoluted tubules (**PCT**), restored distal convoluted tubules (**DCT**).*

Also, the kidneys of control and MLT-treated rats showed presence of basic kidney histology including glomerulus, proximal and distal convoluted tubules and loop of Henle (figure 5A and B). The CLP-treated rats showed presence of widened urinary and congested urinary spaces, congested loop of Henle and loss of glomerular epithelium (Plate 2C-E). However, these lesions were attenuated in the CLP+MLT group (Plate 2 F).

DISCUSSION

This study has shown that cyclophosphamide, an established anticancer agent used for several malignancies is capable of inducing a number of alterations in renal and hepatic functions while the pre-treatment with melatonin, an antioxidant known to be synthesized by the mammalian pineal gland protected against these alterations. Our findings have further corroborated those of previous authors that CLP could cause damage to several tissues since it does not act specifically against cancerous cells but it acts on normal cells and tissues more efficiently, causing damage to them faster than cancerous cells (Bohnenstengel *et al.*, 2000; Christina, 2008; Abdel-Hafez *et al*., 2017).

In the present study, CLP did not induce any significant differences in body and organ weights of the rats. This is in consonant with the reports of Selvakumar *et al*. (2005); Kanno *et al*. (2009); Adikwu and Bokolo (2018). However, a few authors have documented significant difference in body and organ weights due to treatment with CLP in rodents (Kanno *et al*., 2009; El-Naggar *et al*., 2015). These differences may be attributed to the duration and doses of CLP used by these authors. Kanno *et al*. (2009) and El-Naggar *et al*. (2015) used CLP doses as high as 250 and 200 mg/kg, respectively while the present study used 150 mg/kg. Also, while the present study administered rats with a single dose of 150 mg/kg of CLP, Kanno *et al*. (2009) treated mice with CLP doses as high as 250 mg/kg for 7 days.

Cyclophosphamide-treated rats demonstrated hyponatraemia suggestive of altered renal function resulting in reduced excretion of water. This is the direct effect of increased water reabsorption by the collecting ducts of the kidney. Similar findings have been documented in cancer patients undergoing cyclophosphamide therapy (Doshi *et al*., 2012; Salahudeen *et al*., 2014). Also, the CLP induced hypokalaemia observed in this study might be as the result of potassium wasting nephropathy commonly encountered as a major side effect of use of cancer chemotherapeutic agents (Salahudeen *et al*., 2014; El-Naggar *et al*., 2015). The hypochloraemia observed in the CLP-treated rats may be traced to metabolic alkalosis. Hypokalaemia has been associated with metabolic alkalosis especially in the presence of kaliuresis, which occurs in response to low hydrogen ion concentration (Shajani-Yi *et al*., 2016).

The observed CLP-induced significant reductions in the level of serum proteins are suggestive of metabolic imbalances. Altered serum proteins are clinical indicators of toxicities as well as tools needed in the assessment of health status of humans and animals (Sacher and Mcpherson, 2000). In the present study, CLP induced significant increases in the levels of ALP, ALT, AST and GGT compared to the control. This is suggestive of alterations in liver function enzymes of the rats. The CLP-induced significant increases in the levels of AST, ALT and ALP have been documented by El-Nagger

et al. (2015) as well as Shokrzadeh *et al.* (2014). However, the pre-treatment with MLT attenuated the CLP-induced alterations in liver enzymes. This suggests that melatonin is hepatoprotective in CLP-induced liver toxicity. Similar findings on the effect of melatonin have been reported in mice exposed to CLP (Shokrzadeh *et al*.; 2014). Also, propolis, an antioxidizing agent, has also been reported to exert a protective effect against CLP-induced toxicity in the liver of rodents (El-Naggar *et al.*, 2015).

The CLP-induced significant increase in serum creatinine and urea levels compared to the control rats is an indication of alterations in renal function. Similar findings were documented by El-Naggar *et al.* (2015) in mice exposed to single dose of 200 mg/kg of cyclophosphamide. Alterations in serum creatinine and urea levels have been associated with renal diseases especially in cases involving reduced glomerular filtration rate (McWilliam and Macnab, 2009). Interestingly, the pre-treatment with melatonin protected against the observed elevated serum creatinine and urea levels in the rats. This is similar to the report of Abdel-Hafez *et al*. (2017) on the role of melatonin in nephrotoxicity caused by the exposure of rats to anticancer agents.

The hepatic and renal lesions induced by the administration of CLP in rats used in the present study are consistent with our findings on the parameters of hepatic and renal functions. Inflammations as well as vascular congestion of the liver and kidney have been observed in toxicities due to administration of anticancer agents (Rather *et al*., 2016; Abdel-Hafez *et al*., 2017). Moreover, the loss of glomerular epithelium as well as the widened urinary space induced by CLP in the present study is suggestive of altered glomerular function. The protective role of melatonin on the hepatic and renal lesions of rats treated with CLP is similar to that earlier reported by El-Naggar *et al*. (2015) and Abdel-Hafez *et al.* (2017) through the use of propolis and royal jelly, respectively.

Findings from alterations in electrolytes, serum proteins, renal and hepatic function parameters as well as histopathology have shown that CLP is capable of inducing hepatic and renal damage in rats while the pre-treatment with melatonin is able to protect against cyclophosphamide-induced toxicity. Therefore, melatonin is recommended as a pre-treatment supplement in cyclophosphamide therapy.

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