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Research Article

Melatonin enhanced the restoration of biochemical profile in chlorambucil treated-rats: examination of after-withdrawal effects of the drug

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Background: In the wake of global prevalence of different types of cancer, the widespread use of chemotherapy poses threat to the integrity of the reproductive system. Although chlorambucil (Chrm) has anti-calcinogenic action, its administration has been associated with reproductive damage. Similar to chlorambucil, melatonin has anti-cancerous effect. Moreover, the hormone is claimed to protect the reproductive tissues from the insult of different disruptors of their functionality and histoarchitecture. Therefore, the present study investigated the effects of post-administration of melatonin in Chrm treated rats, with an interest in examining the after-withdrawal effects of the drug. **Methods:** Forty rats of ten animals per group were used for the study which lasted for six weeks. The control group received normal saline (vehicle; 0.1 ml/day, *p.o.*) for six weeks, while group 2 was administered saline for three weeks and then Chrm during the subsequent three weeks. However, in groups 3 and 4, Chrm was administered during the first three weeks; thereafter, they were administered saline and melatonin respectively during the subsequent three weeks. Chrm and melatonin were administered at 0.2 and 10 mg/kg b.w./day (*p.o.*) respectively. **Results:** The administration of Chrm significantly decreased gonadotrophin releasing hormone, follicle stimulating hormone, luteinising hormone, testosterone and antioxidant enzymes, but significantly elevated pro-oxidant and pro-inflammatory markers compared to the control group. Moreover, it was accompanied with selected significant alterations of semen parameters and lipid indices. However, restoration of baseline status of testosterone, catalase, total antioxidant capacity, malondialdehyde, lactate dehydrogenase, uric acid, sperm count, and free fatty acid was simply enhanced by the withdrawal of the drug, while that of gonadotrophin releasing hormone, testosterone, semen parameters, superoxide dismutase, catalase, c-reactive protein, lactate dehydrogenase, and high density lipoprotein cholesterol was facilitated by the administration of melatonin. **Conclusion:** The restoration of biochemical profile after chlorambucil treatment could be enhanced by the administration of melatonin.

INTRODUCTION

The International Agency for Research on Cancer (IARC) reported that in year 2018, the global prevalence of the disease had increased by 18.1 million, and that 9.6 million of death was attributed to the disease in the same year. It was estimated that one in six women and one in five men worldwide developed cancer during their lifetime, while one in eleven women and one in eight men die from the disease. Moreover, about 43.8 million of people globally have been guesstimated to be alive within 5 years of cancer diagnosis (International Agency for Research on Cancer, 2018). Therefore, various therapies have been developed for the management of this debilitating disease condition.

Chlorambucil (4-[4-[bis (2-chloroethyl) amino] phenyl] butanoic acid), is an orally available alkylating agent, which has been used especially in the treatment of chronic lymphocytic leukemia and lymphoma (Mahony *et al.*, 1995, Tomenendalova *et al.*, 2008). The drug is primarily metabolised in the hepatic tissue, resulting to the formation of (E)-4-[4 N, N-bis (2.chloroethyl) aminophenyl] 3-butenoic acid (3,4-dehydrochlorambucil) and 2-(4-N, N-bis (2-

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chloroethyl) aminophenyl] acetic acid (phenyl acetic acid mustard) (McLean *et al*., 1980). Chlorambucil and its derivatives form covalent bonds with DNA and proteins of neoplastic cells, causing functional and structural damages to DNA (Pu´ckowska *et al*., 2012). Although the pharmacological agent and its metabolites are cytotoxic to malignant cells, they could also damage normal body cells. The administration of the drug has been associated with hepatotoxicity (Pichon *et al*., 2001), nephrotoxicity (Lameire *et al*., 2011), reproductive toxicity (Delic *et al*., 1986), oxidative stress (Olayinka and Ore, 2014), among others.

Like Chrm, melatonin (N-acetyl-5-methoxytryptamine) has anti-cancerous action (Paroni *et al*., 2014; Alibek *et al*., 2015). Moreover, there are several reports in literature on the antioxidant (Galano *et al*., 2011), anti-inflammatory (Rodrıguez *et al*., 2007), and anti-dyslipidaemic (Esquifino *et al*., 1997) effects of the hormone. Melatonin was observed to have a protective action in testis damaged by chemotherapy (Mohammad *et al*., 2010), testicular torsion (Frungieri *et al*., 2005), and diabetes (Armagan *et al*., 2006). The hormone regulates testicular functions (Reiter 1991) and testosterone secretion (Frungieri *et al*., 2005) by binding to its receptors in the testes. Moreover, melatonin receptors have been found in the hypothalamic neurons, which control the secretion of pituitary gonadotrophins (Wu *et al*., 2006).

Two studies have considered the effects of chlorambucil administration on the reproductive status in experimental animal. Delic and colleagues (Delic *et al*., 1986) observed that the drug caused significant alterations in serum gonadotropins, testosterone, and the morphology of the testicular tissue, while Olayinka and Ore (2014) noted that kolaviron and/or L-ascorbic acid mitigates oxidative stress in the testicular tissue following chlorambucil administration. However, the present study investigated the effects of post-administration of melatonin in Chrm-treated rats, with an interest in examining the after-withdrawal effects of the drug on the reproductive hormones, pro-oxidant/antioxidant indices, inflammatory markers as well as lipid profiles in experimental animal.

METHODS

Drugs and chemicals

Melatonin and chlorambucil were purchased from Sigma chemical company (St. Louis, MO, USA) and Actiza Pharmaceutical Private Ltd (Surat, Gujarat, India) respectively, while sodium pentobarbital was purchased from Nicholas Piramal Ltd (Thane, Maharashtra, India).

Diagnostic kits for the determination of luteinising hormone, prolactin, gonadotrophin releasing hormone, follicle stimulating hormone, testosterone, c - reactive protein and uric acid were obtained from Fortress Diagnostics Limited, UK. Assay kits for the determination of lactate dehydrogenase, malondialdehyde, superoxide dismutase, catalase and total antioxidant capacity were purchased from Elabscience Biotechnology Company Ltd (Wuhan, Hubei, China). The biochemical assays were performed according to the manufacturers' instruction.

Experimental animals and care

Forty (40) male Wistar rats weighing between 200 and 220 g were used for this experiment. They were purchased from the Animal House of Biochemistry Department, University of Ilorin, Nigeria. The rats were kept in plastic cages at a room temperature and photo-periodicity of 30 ^{0}C and 12 hrs light/12 hrs dark respectively. After 14 days of acclimatization, the rats were randomly distributed to separate groups. They were given standard rat chow (manufacturer: Ace Feed PLC, Ibadan, Nigeria) and water *ad libitum* daily, and their weights were measured weekly. The animals were well-catered for in accordance with the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' reported by the National Academy of Sciences (National Academy of Sciences, 2011) and sanctioned by the Ethical Board of the University of Ilorin.

Experimental design

Forty (40) male Wistar rats were used in this study which lasted for six week. They were divided into four (4) groups of ten (10) rats each, which included: group 1 - Control; group 2 – Chlorambucil treated (Chrm); groups 3 – Chrm recovery (Chrm rec); and group 4 - Chrm + Melatonin (Chrm + Mel). The control group was administered normal saline (vehicle, 0.1ml/day; *p.o.*) throughout the duration of the experiment, while group 2 was administered normal saline during the first three (3) weeks and Chrm during the last three (3) weeks of the experiment; however, in groups 3 and 4, Chrm was administered during the first three (3) weeks, afterwards, the rats were administered normal saline (0.1ml/day, *p.o.*) and melatonin respectively during the subsequent three weeks. Chrm tablet was dissolved in normal saline and was administered at 0.2 mg/kg body weight (b.w.)/day (*p.o.*) (Olayinka and Ore, 2014), while melatonin was administered at 10 mg/kg b.w./day (*p.o.*) (Olayaki *et al*., 2018; Adeyemi *et al*., 2019).

Biochemical analyses

Twenty-four (24) hours after administration on the 42nd day of the experiment, the rats were anaesthetised with sodium pentobarbital (40 mg/kg, *i.m.*) (Adeyemi and Olayaki, 2017, 2017a, 2018). Then they were dissected and blood was collected by cardiac puncture into heparinised sample bottles, which were centrifuged at 3500 rpm for 15 min, at – 4 ºC using a cold centrifuge (Benchmark Scientific, Sayreville, USA). The supernatant plasma samples were collected into separate plain bottles prior to the analyses.

Determination of low density lipoprotein cholesterol (LDL-C) level

The formula below was used to determine the low density lipoprotein cholesterol level LDL-C (mg/dl) = TC – (HDL-C - TG/5) (Friedewald *et al*., 1972; Adeyemi and Olayaki, 2019); where TC = total cholesterol; HDL-C = High density lipoprotein cholesterol; TG = Triglyceride

Determination of epididymal sperm parameters

At the end of the experiment, the paired testes of the animals were carefully excised. Thereafter, the epididymal sperm parameters, *viz*; sperm motility, sperm count, sperm morphology, and sperm viability were estimated as described by Salman *et al.* (2014).

Data analyses

Data obtained from the study were analysed using statistical package for social sciences (SPSS) version 18.0 (SPSS Inc., Chicago, Illinois, USA). Statistical evaluations of the group mean differences were tested by one-way analysis of variance (ANOVA) following Tukey *post - hoc* test. The results were expressed as mean ± standard error of mean (SEM), and statistical significance was considered at $p<0.05$.

RESULTS

Effects of chlorambucil (Chrm) with or without melatonin on prolactin, follicle stimulating hormone (FSH), luteinising hormone (LH), gonadotrophin releasing hormone (GNRH), and testosterone

Although there was no significant difference in the prolactin (Fig. 1a) level when comparisons were made among the groups, significant reductions were observed in Chrm, Chrm rec and Chrm + Mel groups, relative to the control group in the FSH (*p* - 0.002, 0.020, 0.022 respectively) (Fig. 1b) and LH (*p* - 0.001, 0.000, 0.000 respectively) results (Fig. 1c). Relative to the latter, significant diminutions in GNRH was only recorded in Chrm (*p* - 0.008) and Chrm rec (*p* - 0.021) groups (Fig. 1d). However, there was a significant increase in GNRH level in Chrm + Mel, compared to Chrm group (*p* - 0.033). Relative to control, Chrm and Chrm rec groups, there was a significant elevation in testeosterone level in Chrm + Mel (*p* - 0.002, 0.000, 0.001 respectively) (Fig. 1e). Moreover, compared to the control group, there was a significant reduction in testosterone in Chrm group (*p* - 0.010). However, relative to the latter, a significant increase in testosterone as noted in Chrm rec group (*p* - 0.016).

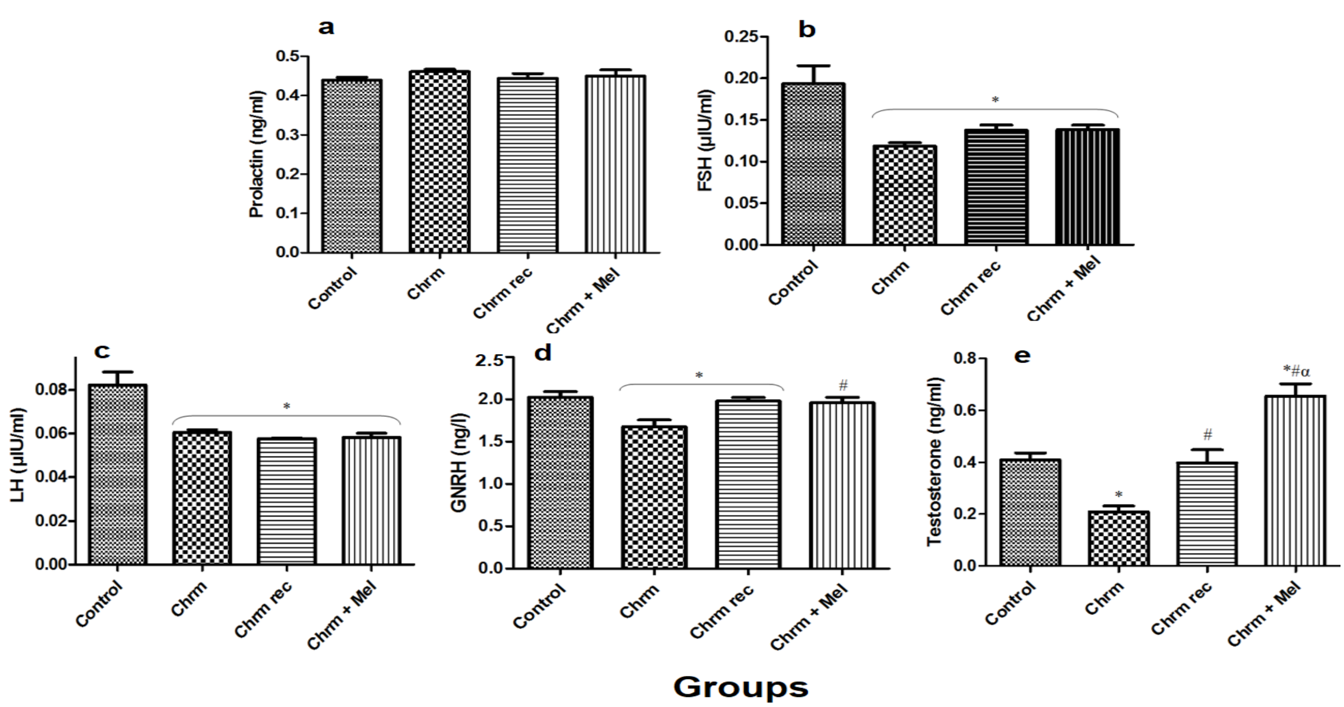

Fig 1: Effects of chlorambucil (Chrm) with or without melatonin on prolactin (Fig. 1a); follicle stimulating hormone (FSH) (Fig. 1b); luteinising hormone (LH) (Fig. 1c); gonadotrophin releasing hormone (GNRH) (Fig. 1d); and testosterone (Fig. 1e) in Wistar rats. Values are expressed as mean ± SEM. $^{*}p < 0.05$ is significant compared to control group; $^{\#}p < 0.05$ is significant compared to Chrm group; $^{\alpha}p < 0.05$ is significant – Chrm rec *vs* Chrm + Mel.

Table 1: Effects of chlorambucil (Chrm) with or without melatonin on antioxidant and inflammatory markers in Wistar rats

Groups/ Parameters	SOD (U/ml)	CAT (Umol/min/ml)	TAC (mM Trolox Equivalent)	LDH (U/L)	MDA (uM)	UA (mg/dl)	CRP (mg/dl)
Control	30.04±3.98	1.45±0.03	4.22±0.63	29.34±0.37	3.24±0.13	10.13±0.19	0.58±0.12
Chrm	4.91±1.25*	1.59±0.02*	1.77±0.04*	54.25±1.16*	3.78±0.13*	12.54±0.83*	4.16±0.24*
Chrm rec	11.77±1.43*	1.48±0.04#	3.15±0.14#	32.51±3.70#	3.40±0.10	10.35±0.40#	3.39±0.10*#
Chrm + Mel	34.94±2.02#α	1.51±0.02	3.13±0.06#	20.81±4.31#α	3.38±0.11	10.02±0.41#	1.35±0.13*#α

Values are expressed as mean ± SEM. *$p < 0.05$ is significant compared to control group; #$p < 0.05$ is significant compared to Chrm group; α$p < 0.05$ is significant – Chrm rec *vs* Chrm + Mel.
NB: SOD – superoxide dismutase; CAT – catalase; TAC – total antioxidant capacity; LDH – lactate dehydrogenase; MDA - malondialdehyde; UA – uric acid; CRP – c - reactive protein

Table 2: Effects of chlorambucil (Chrm) with or without melatonin on semen parameters in Wistar rats

Groups/Parameters	Sperm count ($x10^6$/ml)	Sperm motility (%)	Sperm viability (%)	Sperm morphology (%)
Control	318.40 ± 2.42	69.66 ± 1.77	75.57 ± 2.55	89.84 ± 0.83
Chrm	311.80 ± 3.43	73.78 ± 0.88	74.49 ± 0.66	72.31 ± 0.89*
Chrm rec	350.20 ± 14.24#	70.01 ± 1.17	74.04 ± 1.48	69.55 ± 1.48*
Chrm + Mel	465.40 ± 7.00*#α	82.68 ± 0.28*#α	85.13 ± 0.72*#α	85.71 ± 0.85*#α

Values are expressed as mean ± SEM. *$p < 0.05$ is significant compared to control group; #$p < 0.05$ is significant compared to Chrm group; α$p < 0.05$ is significant – Chrm rec *vs* Chrm + Mel.

Effects of chlorambucil (Chrm) with or without melatonin on catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity (TAC), lactate dehydrogenase (LDH), and malondialdehyde (MDA), uric acid (UA) and c-reactive protein (CRP)

Although there was a significant increase in CAT activity in Chrm group, relative to the control (*p* - 0.009), and a significant reduction in the enzyme activity in Chrm rec compared to the former (*p* - 0.050) (Table 1), nevertheless, significant diminutions in the activity of SOD was recorded in Chrm and Chrm rec compared to the control group (*p* - 0.000, 0.000 respectively). However, the level of activity of SOD was significantly increased in Chrm + Mel relative to Chrm (*p* - 0.000) and Chrm rec (*p* - 0.000) groups. A significant reduction in TAC was noted in Chrm compared to the control group (*p* - 0.000); however, relative to the latter, there were significant elevations in LDH and MDA in Chrm group (*p* - 0.000, 0.025 respectively). In the Chrm rec and Chrm + Mel groups, compared to Chrm group, there were significant elevations in TAC (*p* - 0.038, 0.041 respectively), but significant reductions in LDH (*p* - 0.000, 0.000 respectively). Also, relative to Chrm rec, there was a significant decrease in the activity of LDH in Chrm + Mel group (*p* - 0.050) (Table 1).

In comparison to the control group, there was a significant increase in the plasma level of UA and CRP in Chrm group (*p* - 0.020, 0.000 respectively). Moreover, CRP was significantly increased in Chrm rec and Chrm + Mel groups, compared to the control (*p* - 0.000, 0.014 respectively) (Table 1). Relative to Chrm group, there were significant reductions in Chrm rec and Chrm + Mel groups in the UA (*p* - 0.037, 0.015 respectively) and CRP (*p* - 0.013, 0.000 respectively) results. Moreover, a significant decrease in the level of CRP was recorded in Chrm + Mel, compared to Chrm rec group (*p* - 0.000) (Table 1).

Effects of chlorambucil (Chrm) with or without melatonin on semen parameters

Relative to control, Chrm and Chrm rec groups, there were significant increases in sperm count (*p* - 0.000, 0.000, 0.000 respectively), sperm motility (*p* - 0.000, 0.000, 0.000 respectively) and sperm viability (*p* - 0.003, 0.001, 0.001 respectively) in Chrm + Mel group (Table 2). A significant increase in the sperm count was noted in Chrm rec relative to Chrm group (*p* - 0.021). Compared to Chrm + Mel, significant decreases in sperm morphology were observed in Chrm (*p* - 0.000) and Chrm rec (*p* - 0.000) groups. Sperm morphology was recorded to be significantly increased in the control group compared to Chrm (*p* - 0.000), Chrm rec (*p* - 0.000) and Chrm Mel (*p* - 0.050) groups (Table 2).

Table 3: Effects of chlorambucil (Chrm) with or without melatonin on lipid indices in Wistar rats

Groups/ Parameters	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	High density lipoprotein cholesterol (mg/dl)	Low density lipoprotein cholesterol (mg/dl)	Free fatty acids (mg/dl)	Phospholipids (mg/dl)
Control	59.46 ± 1.98	41.58 ± 1.03	8.22 ± 0.77	59.46 ± 1.98	822.26 ± 67.44	50.46 ± 1.67
Chrm	57.18 ± 0.76	46.24 ± 0.69*	3.50 ± 0.30*	57.18 ± 0.76	1208.30 ± 100.32*	41.04 ± 3.34
Chrm rec	56.71 ± 0.42	47.08 ± 0.86*	4.45 ± 0.27*	56.91 ± 0.47	751.43 ± 67.22#	46.36 ± 3.20
Chrm+Mel	58.86 ± 1.84	47.16 ± 1.72*	8.22 ± 0.73#α	58.86 ± 1.84	742.55 ± 27.74#	39.08 ± 6.46

Values are expressed as mean ± SEM. **p* < 0.05 is significant compared to control group; #*p* < 0.05 is significant compared to Chrm group; α*p* < 0.05 is significant – Chrm rec *vs* Chrm + Mel.

Effects of chlorambucil (Chrm) with or without melatonin on lipid indices

There was no significant difference in the plasma levels of TC, LDL-c and phospholipids when comparisons were made across the groups (Table 3). However, relative to the control group, there were significant increases in TG in Chrm (*p* - 0.048), Chrm rec (*p* - 0.017) and Chrm + Mel (*p* - 0.016) groups, significant reductions in HDL-C in Chrm (*p* - 0.000) and Chrm rec (*p* - 0.001), and significant increase in FFA in Chrm group (*p* - 0.007). Moreover, compared to Chrm group, there were significant reductions in FFA in Chrm rec (*p* - 0.002) and Chrm + Mel (*p* - 0.001) groups, and a significant increase in HDL-C in Chrm + Mel group (*p* - 0.000). In addition, the level of HDL-C in the latter was recorded to be significantly increased compared to Chrm rec group (*p* - 0.001) (Table 3).

DISCUSSION

The results of the present study showed that chlorambucil caused imbalance in hormonal profile in the hypothalamic-pituitary-gonadal axis, evident by the findings of semen indices. Moreover, the drug precipitated oxidative stress, dyslipidaemia and pro-inflammatory events in rats. Nevertheless, there was fair reversal of biochemical profile and testicular histoarchitecture to the basal status after the stoppage of chlorambucil administration. Restoration of some biomarkers, but not the testicular integrity to the homeostatic state was facilitated by intervention with melatonin.

In this study, the neurotoxic effects of chlorambucil was found to be prolactin-independent, however, the drug caused significant reduction in the endogenous levels of GNRH, FSH and LH. Although melatonin receptors have been characterised in the pituitary (Vanecek *et al.*, 1987), exogenous administration of the hormone was found to have no effect on the plasma levels of gonadotrophins. Chlorambucil administration was accompanied with a significant decrease in testosterone level (Delic *et al.*, 1986). This is no doubt associated with the reduction of LH, which stimulates the interstitial cells of leydig to secrete testosterone. After the stoppage of administration of Chrm, there was restoration of testosterone to the baseline level, even though corresponding effect was not recorded in the estimated GNRH, FSH and LH. Chlorambucil is known to have a neurotoxic action; nevertheless, the exact mechanism has not been clearly established (Wolfson and Olney, 1957; Salloum *et al.*, 1997). Post-treatment with melatonin after chlorambucil administration caused a significant increase in testosterone level. This could be attributed to the action of the melatonin on its receptors in the testes, by which the hormone regulates testicular functions (Reiter, 1991) and testosterone secretion (Frungieri *et al.*, 2005). Chlorambucil was observed to have no effect on sperm motility and viability. However, its effect was expressed in the sperm morphology, with an evidence of the reversal of the action of the drug on the sperm count. Significant increase in sperm count in the Chrm rec group compared to Chrm treated group, which was partly supported by the histological presentations, could be ascribed to the significant elevation in level of testosterone in group 3 (Chrm rec) compared to group 2 (Chrm treated) and endogenous compensatory mechanisms after the withdrawal of the drug. Despite the observed disparity in sperm count in group 3 relative to group 2, the sperm motility, sperm viability and sperm morphology does not reflect the same pattern. Surprisingly, melatonin was noted to cause significant increases in all the semen indices, even though the hormone had no significant effect on the pituitary gonadotrophins and structural presentation of the testicular tissue. This effect is no doubt related to the stimulatory action of the hormone on the uncompromised sperm producing cells in the testicular tissue post-administration of chlorambucil and the effect of the hormone on GNRH and testosterone secretions. The latter is well-known to be required in virtually every stage of gametogenesis.

Sperm cells are especially prone to ROS-induced damages, because they don't have DNA repair mechanisms. Moreover, they possess minimal levels of antioxidant enzymes and elevated levels of polyunsaturated fatty acids (Lewis and Aitken, 2005; Agarwal *et al*., 2008). Nonetheless, small amounts of ROS are crucial for spermatozoa to acquire fertilising status (Aitken, 1999). In the present study, the administration of chlorambucil was characterised by imbalance of the antioxidant enzyme system - an event that has been associated with oxidative stress and hence lipid peroxidation. Although there was no significant difference in the activity of SOD in Chrm vs Chrm rec, the reversal of the effects of the drug on oxidative process post-administration was indicated by the CAT, TAC, LDH and MDA results. Melatonin showed no significant effect on these parameters probably because the threat posed by chlorambucil on the antioxidant system did not persist. However, the hormone caused significant changes in SOD and LDH, relative to what was recorded in Chrm and Chrm rec groups. Melatonin and its metabolites are powerful scavengers of oxygen and nitrogen free radicals (Manda *et al*., 2007), and as such, they could inhibit lipid peroxidation and hence cellular damage. Elevated status of LDH was observed to correspond with an increase in the level of MDA (the product of lipid peroxidation) in our previous studies (Adeyemi and Olayaki, 2018a; Adeyemi and Olayaki, 2018b). The enzyme has been tagged an indicator of tissue damage (Shi *et al*., 2003).

Inflammatory events are associated with oxidative stress. Compromised oxidative status is often accompanied with elevated inflammatory markers (Ige *et al.* 2012; Adeniyi *et al.* 2016; Olayaki *et al*., 2018a; Adeyemi and Olayaki, 2018c, 2019a). The pathways that activate the production of inflammatory mediators are all prompted by oxidative stress (Haddad, 2002). There were significant reductions in the plasma levels of UA and CRP in Chrm rec group, following an initial elevation in the Chrm group. Melatonin was observed to have significant effect on UA but not CRP. The administration of the hormone has been documented to be accompanied with reductions in pro-inflammatory cytokines (Rodrıguez *et al*., 2007). The UA result corresponded with that of the TAC, while that of the CRP closely mimicked that of LDH. These of course affirmed the connectedness between oxidative and inflammatory events.

Male reproductive dysfunction has not only been linked with hormonal imbalance, oxidative stress and inflammation, but also, dyslipidaemia. Cholesterol and lipid homeostasis are imperative for male fertility (Cross, 1998; Maqdasy *et al*., 2013). About 65% of infertile men were observed to be challenged by triglyceridaemia and/or hypercholesterolaemia (Ramírez-Torres *et al*., 2000). Comparisons across the groups revealed that there was no significant difference in the total cholesterol, LDL-c and phospholipids; however, the sustained effects of chlorambucil on TG and HDL-c after the stoppage of administration showed that the drug might have altered lipid metabolism. Nevertheless, the FFA level in Chrm rec vs Chrm further substantiated the claim that the toxic effects of chlorambucil could be partially reversed after the stoppage of administration. Esquifino and colleagues opined that the anti-dyslipidaemic effect of melatonin may be associated with its ability to enhance lecithin-cholesterol acyltransferase (LCAT)-mediated cholesterol esterification (Esquifino *et al*., 1997). This was evidenced by the significant decreases in HDL-C and FFA in Chrm, compared to Chrm + Mel group.

CONCLUSION

Restoration of biochemical profile after chlorambucil treatment could be enhanced by the administration of melatonin.

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L.A.O. and W.J.A. ('co-first authors') conceived and supervised the study. W.J.A. did the statistical analysis and wrote the initial and final drafts of the manuscript. E.A., O.O., I.B., and S.J. carried out the experiments and provided funding for the study.

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