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Melatonin Alleviates Constant Light Induced Oxidative Stress Damages of Kidney and Spleen of Female Rat

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Abstract: Photoperiod refers to the relative duration of light and dark that an animal experiences over the course of a 24 h period. Light is the principal stimulus that causes profound physiological and behavioural changes in all living organism. By manipulating photoperiod, melatonin production changes and its secretion drives enduring changes in many physiological systems of our body. Taking into consideration that light can affect pineal function and melatonin can be used as reactive oxygen species scavenger, act as a potent antioxidant and immunomodulator in mammals, the present experiment was designed and conducted to explore the consequences of constant light exposure on oxidative stress and antioxidant status on kidney and spleen of rat. 16 young adult female rats were randomly divided into 3 groups. The 4 rats of first group were employed as Control. These animals were kept under a 12 h light (600 lux, 60 days)/12 h dark cycle. The 8 rats of second group were maintained under constant light condition (600 lux, 60 days). After 60 days of constant light exposure second group of rats were randomly divided into two. One of these group of rats were designated as constant light exposed rats (LL) and another group of rats exposed to constant light is treated with melatonin (LL+MEL) for 21 days. The 4 rats of third group were only supplemented melatonin (MEL). The results showed that constant light exposure (60 days, 600 lux) resulted in an increased lipid peroxidation rate in kidney and spleen tissue and could effectively disturb the immune cells function through excessive generation of free radicals and decreased antioxidant enzymes activity (superoxide dismutase, catalase, glutathione peroxidase) through physiological or functional pinealectomy. Exogenous melatonin administration (200 µg/100 g body weight/day) suppressed the constant light induced oxidative stress and increase the activity of antioxidant enzymes in kidney and spleen of rat. Hence, melatonin has a nephroprotective and immunoprotective effects following photoperiodic stress induced cellular damages in female rat model.

Keywords: Melatonin, Photoperiod, Antioxidant, Immunomodulator, Superoxide dismutase, Catalase, Glutathione peroxidase, Kidney, Spleen, Oxidative stress

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Introduction

Photoperiod is among the many environmental cues that control circadian rhythms which may modify many of the physiological and behavioral responses in all living organisms. The differential proportion of light and dark that an animal encounters across the 24 h is referred to as photoperiod (Wankhade *et al*., 2019) which is so-called "biological calendar" (Nelson *et al*., 2010). Melatonin, in particular, is a key hormone that plays a major role in the photoperiod-induced physiological changes (Elsabagh *et al*., 2020). Melatonin production is altered by photoperiod, attaining both its maximum level and minimum level at the mid- point of the dark phase and the mid-point of the light phase, respectively.

The melatonin (N-acetyl-5-methoxytrypt-amine) is a neurohormone, which functions as a photoperiod transducer, which is primarily synthesized and released by the pineal gland at night (Baekelandt *et al*., 2020). The pineal indole amine was discovered and isolated from bovine pineal by Aaron Lerner, which he named "MELATONIN" (Lerner *et al*., 1958). The daily and seasonal variations in the environmental Light-Dark cycle play a critical role in controlling melatonin biosynthesis within the pineal gland. Melatonin presently has a much wider range of applications than was initially anticipated, both in terms of its widespread distribution and functional variety. In particular, as a result of conclusive investigations conducted during the last 15 years, melatonin has been connected to a wide range of functions, including anti-inflammation, antioxidant, oncostatic, circadian rhythm control, immune function modulation etc (Reiter *et al*., 2014). In addition to pineal gland, 25% of melatonin is produced from retina, bone marrow cells, platelets, skin, lymphocytes, harderian gland, cerebellum and especially in gastrointestinal tract of vertebrates (Hardeland *et al*., 2005; Acuna *et al*., 2014).

Since over 30 years ago, melatonin has been recognised to directly scavenge free radicals (Tan *et al*., 1993). Being amphiphilic molecule, melatonin rapidly crosses the plasma membrane lipid barrier and functions as a strong antioxidant within the antioxidative defence system (Tan *et al*., 2002). After already being identified as a direct free radical scavenger, melatonin was discovered to activate antioxidant enzymes such glutathione peroxidase and glutathione reductase (Barlow *et al*., 1995). Melatonin successfully combats oxidative stress by direct scavenging of reactive oxygen species (ROS) and indirectly by upregulation of antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GP) and down regulation of pro-oxidant enzymes such as nitric oxide synthase (NOS) and lipoxygenase (Reiter *et al*., 2016). In a number of human experiments, melatonin has been successfully utilised to minimize oxidative stress and cellular apoptosis as well as to restore tissue function; this success justifies its broader usage in a wider range of human investigations. These chronobiotic molecule have been shown to have a variety of beneficial effects, including immune regulation in rodents that breed seasonally (Haldar *et al*., 2004), immunoprotective (Rai *et al*., 2013) effects that are mediated by the endocrine systems (Hardeland *et al*., 2005; Lahiri *et al*., 2006). It is also regarded as neuroprotective in medicine (Cardinali *et al*., 2013). Earlier findings of Rai *et al*. (2016) reported the hepato-renal protective action of melatonin. However, melatonin has well-known cytoprotective effects via paracrine or autocrine mechanisms.

The present experimental study was designed and conducted to obtain information about the constant light induced alterations in different biomarkers of oxidative stress and how it affects immune and renal functioning of spleen and kidney, respectively of a female albino rat followed by the exogenous administration of melatonin which aimed to evaluate the protective role of melatonin as a nephroprotective and immunoprotective due to its potent antioxidative properties.

Materials and Methods

All of the experimental procedures with animals and their maintenance were performed in accordance with national guidelines and regulations for care and use of laboratory animals of institutional practice of Institutional Animal Ethics Committee (IAEC), GGU, Bilaspur (C.G.) India. Sixteen healthy female albino rats (wistar strain) of approximately same age weighing 170±10 g were procured from Columbia Institute of Pharmacy, Raipur. Upon arrival the rats were weighed, group-housed and kept under standard laboratory conditions. Rats were provided with foods and water *ad libitum*. After an acclimatization period of two weeks rats were used for experiment.

Experimental Design:

Group 1 Control: Received 0.1% normal saline (n=4).

Group 2 Constant light (LL) group: Received 600 lux light, 60 days (n=4)

Group 3 Constant light (LL) + Melatonin (MEL) group: Received 600 lux light + melatonin 200 µg/100 g body weight/day (n=4)

Group 4 Melatonin (MEL) group: Received 200 µg/100 g body weight/day of melatonin (n=4).

After two weeks of acclimatization 16 young adult female rats were randomly divided into 3 groups. The 4 rats of first group were designated as normal (Control). They were housed under natural laboratory condition and do not undergo any experimental manipulations. Specifically, these animals were kept under a 12 h light (600 lux, 60 days)/12 h dark cycle. The 8 rats of second group were maintained under constant light condition (600 lux, 60 days). After 60 days of constant light exposure second group of rats were randomly divided into two. One of these group of rats were designated as constant light exposed rats (LL) and another group of rats exposed to constant light is injected with melatonin supplementation (LL+MEL) for 21 days. The rest 4 rats of group were supplemented with only melatonin (MEL).

Chemicals and Reagents:

Sodium Carbonate, Sodium Hydroxide, Sodium Potassium Tartarate, Copper Sulphate, Folin's Reagent, Bovine Serum Albumin (BSA), DNTB, Methanol, Hydrochloric acid (HCL), Phenazine methosulphate, Nitro-Blue Tetrazolium (NBT), Glacial Acetic Acid, Butylated Hydroxytoluene (BHT), Thio Barbituric acid (TBA) (0.6%), Phosphoric Acid and melatonin were purchased from CDH (Local Supplier). Hydrogen Peroxide Phosphate Buffer, Tris Buffer (pH - 8.2), Sucrose Buffer (pH - 8.2), Phosphate Buffered Saline (pH - 7.4), Sodium Pyrophosphate Buffer, NADH, Phosphate Buffer (pH - 7.4) and Tris-HCl Buffer (pH 7.4) were prepared for performing different biochemical protocols.

Drugs and treatment:

Melatonin solution was prepared by dissolving it in few drops of ethanol and then diluting it with normal saline (0.1% saline) up to the appropriate concentration. The control (CON) rats were given 0.1% normal saline/100 g body weight/day. One of groups of continually light exposed rats were injected with melatonin supplementation (200 µg/100 g body weight/day). Following complete anaesthesia rats of each group were scarified. In order to observe the effect of constant light exposure and melatonin the kidney and spleen were dissected out and subjected to weight analysis, malondialdehyde assay to measure lipid peroxidation level and different parameters of oxidative stress.

Parameters studied (*kidney and spleen*):

Gravimetric Analysis:

The kidney and spleen were removed from adjacent connective tissue. The weight of kidney and spleen were measured using the weighing balance to observe changes in the relative weight of spleen and kidney of different experimental groups on induction of oxidative stress due to

constant lighting condition and after treatment of melatonin in order to compare with control.

Protein Estimation (*Lowry's Method, 1951*):

Solution A: Firstly, the solution A was prepared by mixing sodium carbonate solution (dissolved in distilled water) and copper sulphate solution (dissolved in distilled water).

Solution B: It was prepared by dissolving sodium potassium tartrate in distilled water.

Solution C: It was freshly prepared by mixing Solution A and Solution B in equal amounts.

Fresh tissue was taken and homogenised. 0.2 ml of 10% TCA was added to the homogenate and centrifuged at 2000 rpm for 15 min. pellet was discarded and to the supernatant 1 ml of NAOH (0.2N) was added. From the above prepared sample solution 0.1 ml was pipette out, to this 0.4 ml of distilled water was added. 5 ml of solution C was added and mixed well. Then Sample solution was incubated for 10 min at room temperature. 0.5 ml of Folin's reagent was added to the reaction mixture and incubated for 30 min at room temperature to develop colour and absorbance was measurede at 625 nm.

Lipid Peroxidation (*LPO*) *Assay* (*Ohkawa et al.*, *1979*):

During oxidative cell membrane damage, thiobarbituric acid reactive compounds (TBARS) are created. Malondialdehyde (MDA), one of the main by-products of lipid breakdown, is a widely-used parameter to measure lipid peroxidation. The spleen and kidney were excised and 100 mg of fresh tissue was taken and homogenate was prepared by adding 1 ml of 20 mM Tris-HCl buffer (pH 7.4) to it. Equal amounts of homogenate and BHT were taken and 3 ml of phosphoric acid and 1 ml of phosphoric acid and 1 ml of TBA was mixed to it. Reaction mixtures were heated at 95℃ in water bath for 45 min and then the sample solution was cooled at room temperature and centrifuged at 3000 rpm for 15 min. Only supernatant was taken and pellet were decanted. optical density of supernatant was measured at 535 nm. Lipid peroxidation was expressed in nmol TBARS formed/mg protein of experimental tissues.

Superoxide Dismutase Activity Assay (*Kakkar et al.*, *1984*):

Fresh tissue was taken and homogenised in 1ml phosphate buffer saline (PBS, pH-7.4). 0.2 ml of homogenate was taken in eppendorf and centrifuged at 1500 rpm for 10 min and only supernatant was taken and pellet were discarded. Then 1.2 ml of sodium pyrophosphate buffer, 0.1 ml 0f PMS (phenazine methosulphate) and 0.3 ml of NBT (Nitro blue tetrazolium) were added to the supernatant and mixed well. 0.2 ml of NADH was added to above solution and then 1 ml of glacial acetic acid was mixed. Finally, absorbance was measured at 560 nm.

Catalase Activity Assay (*Beers and Sizer*, *1952*):

The homogenate was prepared in phosphate buffer. Phosphate buffer (pH 8.2) with volume of 2.5 ml was added to 0.1 ml of homogenate and centrifuged at 3000 rpm for 15 min. 40 µl of supernatant was taken and 3 ml of H_2O_2 phosphate was added to it and mixed thoroughly and then absorbance was measured at 240 nm. The activity of CAT was expressed as the amount of H_2O_2 degraded per minute.

Reduced Glutathione (*GSH*) *Assay*:

100 mg of fresh tissue was taken and homogenised in 1 ml of sucrose buffer and centrifuged at 3000 rpm for 15 min. Only supernatant was taken and pellet were decanted. To 100 µl of supernatant 1.5 ml of Tris Buffer (pH 8.2), 0.1 ml of DTNB was added to it. The volume was made up to 10 ml by methanol and vortexed. The sample solution was incubated at room temperature for 30 min and then centrifuged at 3000 rpm for 15 min and absorbance was measured at 412 nm.

Statistical analysis:

Results were expressed as Mean±SE. Data were analysed by performing one way ANOVA through SPSS (26.0) version software followed by Tukey's

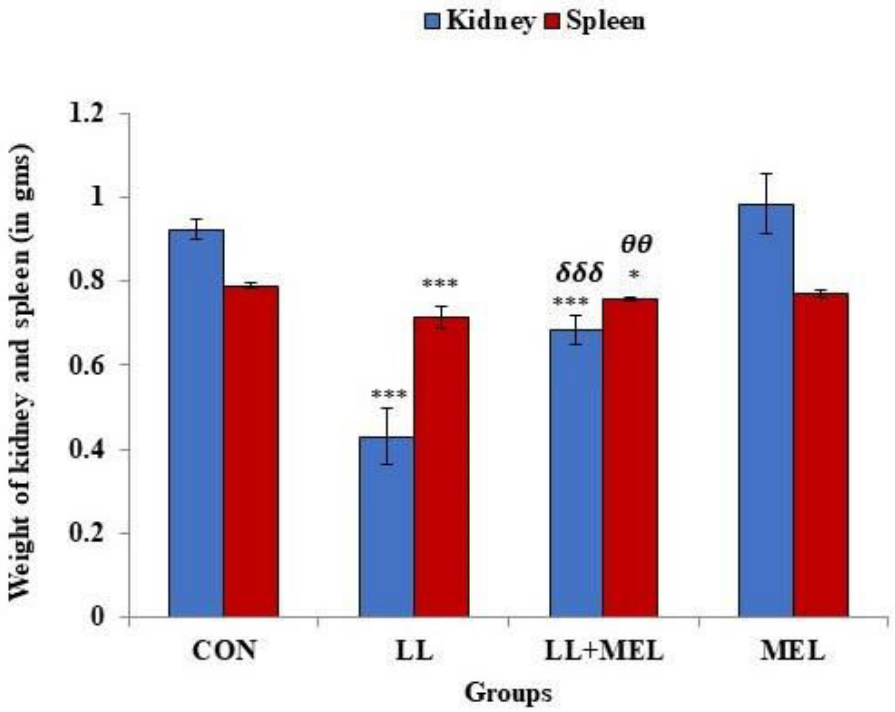

Fig. 1: Effect of constant light exposure and exogenous melatonin on weight of kidney and spleen. Histogram represent Mean ± SE; N=4; CON = Control, LL = Constant light (600 lux, 60 days), LL + MEL = Light + Melatonin (200 µg/100 g body weight/day), MEL = Melatonin. Histogram represents */***δ***/***θ*** P≤0.05, **/***δδ***/***θθ*** P≤0.01 and ***/***δδδ***/***θθθ*** P≤0.001, where * represents CON vs. LL, ***δ*** represents LL vs. LL + MEL of kidney and ***θ*** represents LL vs. LL + MEL of spleen.

t-test. Data were considered at P ≤ 0.05, P ≤ 0.01, P ≤ 0.001.

Results

Effect of constant light exposure and melatonin on weight of kidney and spleen:

In gravimetric analysis, effect of constant light exposure caused a significant decrease in relative kidney and spleen weight in comparison with control rat. Treatment of continual light exposed rats with exogenous melatonin improved the relative kidney and spleen weight in comparison with the kidney and splenic weight of constant light (LL) group rats (Fig. 1).

Effect of constant light exposure and melatonin on protein content:

Kidney and spleen from experimental rats exposed to constant light showed a significantly lower protein content in comparison with kidney and spleen of control rat. Exogenous melatonin supplementation showed a significant rise in protein content of the kidney and spleen in rats that were continuously exposed to light (Fig. 2).

Effect of constant light exposure and Melatonin on lipid peroxidation (*LPO level*):

Malondialdehyde (MDA) is the last product of lipid breakdown formed by oxidative stress. MDA is an authentic marker for lipid peroxidation and it was analysed in reference to TBARS formed per mg protein of kidney and spleen. The MDA level was higher in kidney and spleen tissue of rat exposed to constant lighting condition in comparison with kidney and spleen of control rat. Administration of melatonin effectively and significantly lowered the MDA level in kidney and spleen of constant light exposed rats (Fig. 3).

Effect of constant light exposure and melatonin on catalase enzyme activity (*CAT*):

Catalase is a key antioxidant enzyme that catalyses the breakdown of H_2O_2 formed in the living tissues. Exposure of constant light significantly suppressed the catalase activity in the kidney and

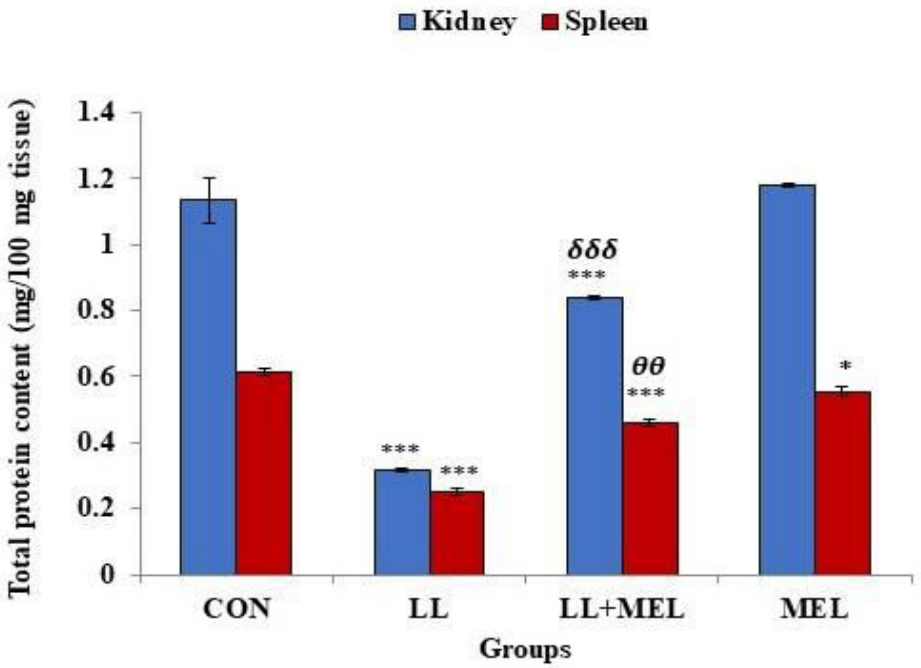

Fig. 2: Effect of constant light exposure and exogenous melatonin on protein content in kidney and spleen. Histogram represent Mean ± SE; N=4; CON = Control, LL = Constant light (600 lux, 60 days), LL + MEL = Light + Melatonin (200 µg/100 g body weight/day), MEL = Melatonin. Histogram represents */***δ**/**θ** P≤0.05, **/***δδ*/***θθ*** P≤0.01 and ***/***δδδ*/***θθθ*** P≤0.001, where * represents CON vs. LL, ***δ*** represents LL vs. LL + MEL of kidney and ***θ*** represents LL vs. LL + MEL of spleen.

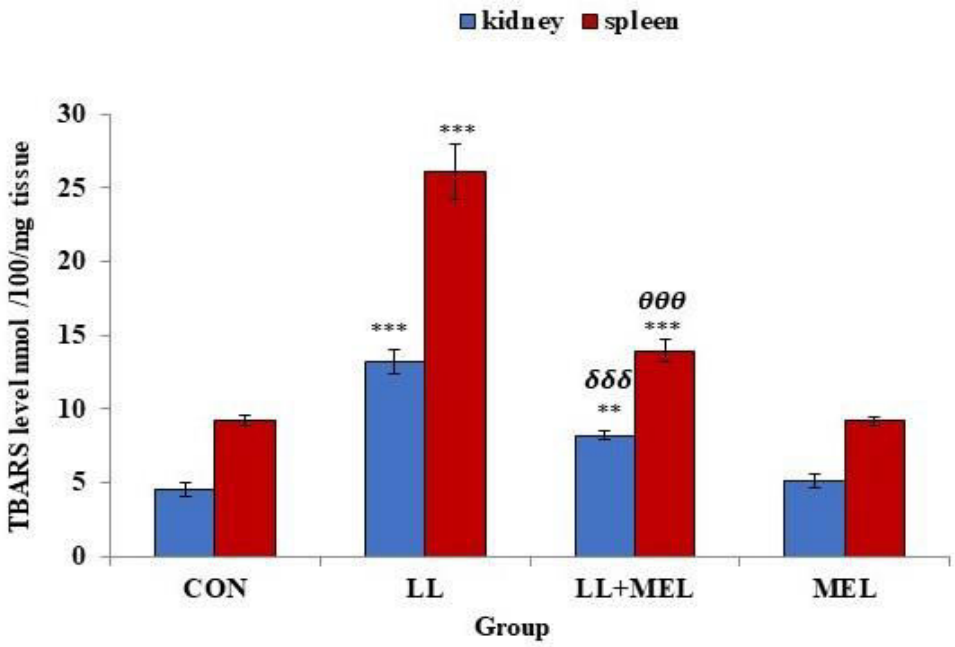

Fig. 3: Effect of constant light exposure and exogenous melatonin on lipid peroxidation (LPO) level in kidney and spleen. Histogram represent Mean ± SE; N=4; CON = Control, LL = Constant light (600 lux, 60 days), LL + MEL = Light + Melatonin (200 µg/100 g body weight/day), MEL = Melatonin. Histogram represents */***δ**/**θ** P≤0.05, **/***δδ*/***θθ*** P≤0.01 and ***/***δδδ*/***θθθ*** P≤0.001, where * represents CON vs. LL, ***δ*** represents LL vs. LL + MEL of kidney and ***θ*** represents LL vs. LL + MEL of spleen

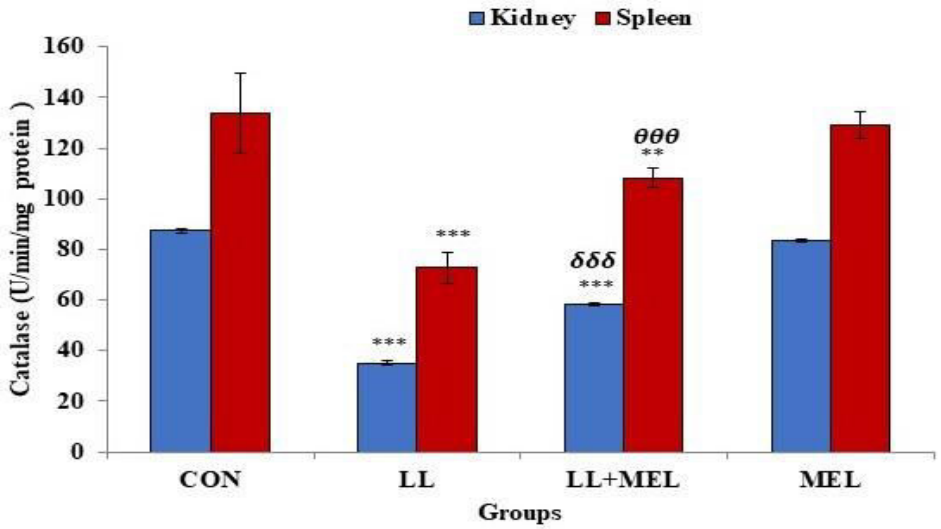

Fig. 4: Effect of constant light exposure and exogenous melatonin on Catalase (CAT) activity in kidney and spleen. Histogram represent Mean ± SE; N=4; CON = Control, LL = Constant light (600 lux, 60 days), LL + MEL = Light + Melatonin (200 µg/100 g body weight/day), MEL = Melatonin. Histogram represents */***δ***/***θ*** P≤0.05, **/***δδ***/***θθ*** P≤0.01 and ***/***δδδ***/***θθθ*** P≤0.001, where * represents CON vs. LL, ***δ*** represents LL vs. LL + MEL of kidney and ***θ*** represents LL vs. LL + MEL of spleen.

splenic tissue in comparison with the kidney and spleen of control rat (normal). Melatonin supplemented to constant light (LL) group rat significantly increased the catalase enzyme activity in comparison with continual light exposed rats (Fig. 4).

Effect of constant light exposure and melatonin on SOD enzyme activity:

Superoxide dismutase (SOD) is an important antioxidant enzyme that is responsible to neutralize superoxide anion formed in living organisms. A significant decrease in SOD enzyme activity was observed in constant light administered rat in comparison with the kidney and spleen of controlled rat. Melatonin treatment elevated the superoxide dismutase enzyme activity significantly in comparison with the kidney and spleen of constant light (LL) group rats (Fig. 5).

Effect of constant light exposure and melatonin on reduced glutathione level (*GSH*):

Glutathione is a crucial antioxidant and by donating an electron, it reduces hydroperoxides to their corresponding alcohols in living organisms. Exposure of constant light significantly decreased the reduced glutathione level in kidney and spleen tissue in comparison with the kidney and spleen of control rat. Treatment of rats with exogenous melatonin significantly increased the reduced glutathione level in kidney and spleen of continual light exposed rats (Fig. 6).

Discussion

The present study demonstrated that exposure of constant light induced oxidative stress in various organ of the body, including the immune modulator organ spleen (El-Bakry *et al*., 2018) and nephron-protective organ kidney (Rai *et al.,* 2016) of experimental rats by generating highly reactive oxygen species. In this study suppression of melatonin secretion from pineal gland leads to a variety of negative effects, including oxidative stress, kidney dysfunction and immune organ impairments (Nelson *et al*., 2007). It is evident from the present and previous experimental

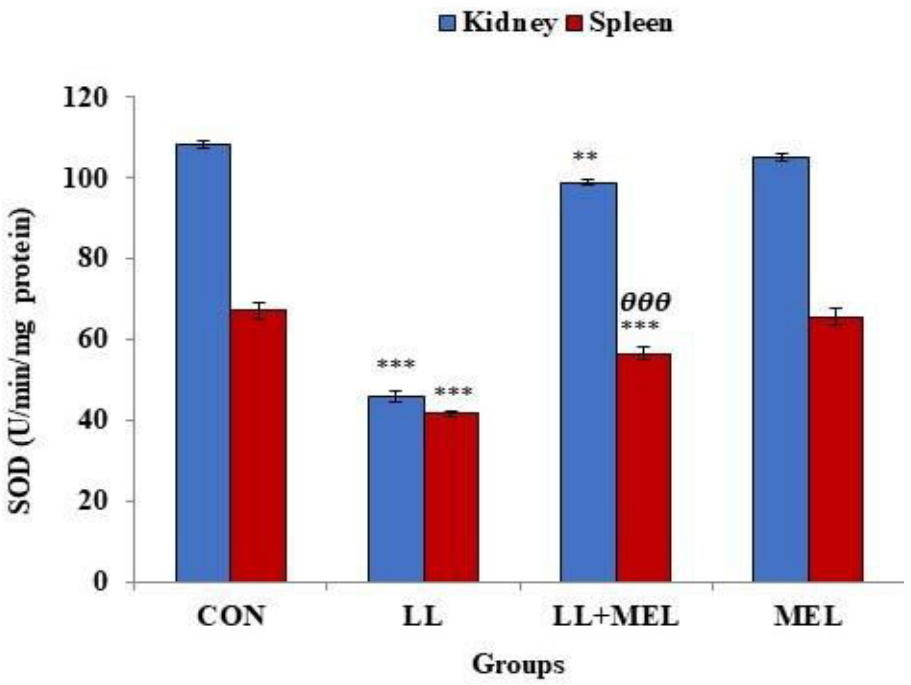

Fig. 5: Effect of constant light exposure and exogenous melatonin on superoxide dismutase (SOD) activity in kidney and spleen. Histogram represent Mean ± SE; N=4; CON = Control, LL = Constant light (600 lux, 60 days), LL + MEL = Light + Melatonin (200 µg/100g body weight/day), MEL = Melatonin. Histogram represents */***δ**/**θ** P≤0.05, **/***δδ*/***θθ*** P≤0.01 and ***/***δδδ*/***θθθ*** P≤0.001, where * represents CON vs. LL, ***δ*** represents LL vs. LL + MEL of kidney and ***θ*** represents LL vs. LL + MEL of spleen.

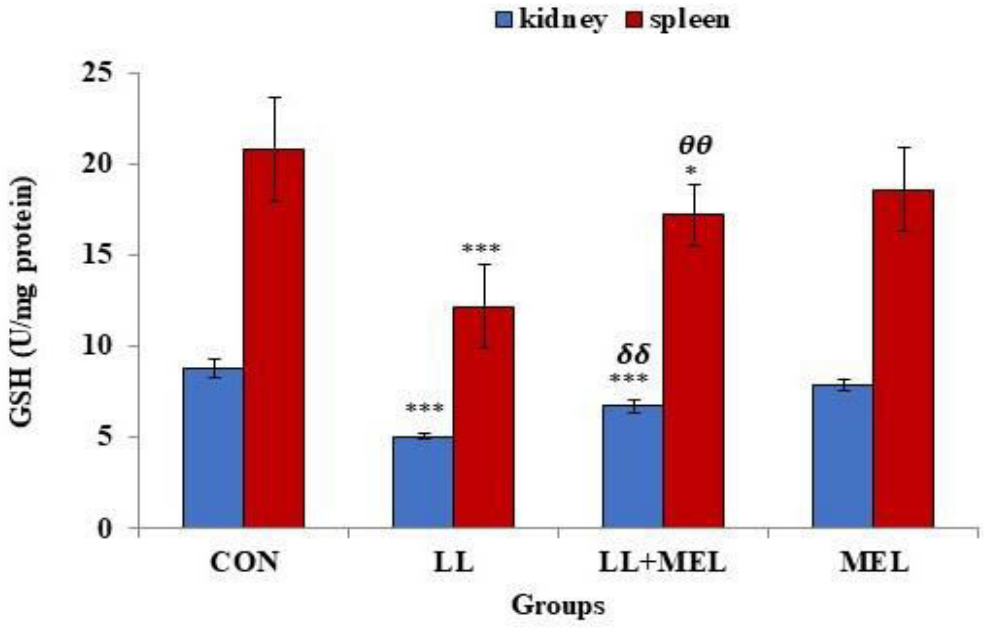

Fig. 6: Effect of constant light exposure and exogenous melatonin on Reduced glutathione (GSH) activity in kidney and spleen. Histogram represent Mean ± SE; N=4; CON = Control, LL = Constant light (600 lux, 60 days), LL + MEL = Light + Melatonin (200 µg/100g body weight/day), MEL = Melatonin. Histogram represents */***δ**/**θ** P≤0.05, **/***δδ*/***θθ*** P≤0.01 and ***/***δδδ*/***θθθ*** P≤0.001, where * represents CON vs. LL, ***δ*** represents LL vs. LL + MEL of kidney and ***θ*** represents LL vs. LL + MEL of spleen

studies that the pro-oxidative effects of constant light could be prevented through the administration of melatonin (Baydas *et al*., 2001). This statement points toward the association between diminished antioxidative activities and suppressed melatonin due to constant light exposure. Melatonin acts as a free radical scavenger, so it is considered as a major element of the antioxidant defense system and it is also able to increase the expression or the activity of the main antioxidant enzymes hence act as an indirect antioxidant (Hardeland *et al.,* 2006).

Constant light exposure resulted in a significant decrease on weight of kidney and spleen which may reduce the renal and immune functioning of these crucial organs. Exogenous administration of melatonin significantly increased the relative weight of kidney and spleen. Our result also supports the pro-oxidative effect of constant light in spleen and kidney to exacerbate the oxidative stress when the LPO level was noted significantly high which might have reduce the renal and immune functions as well as proved the potentially antioxidative property of melatonin (Rai *et al*., 2009). Recent research has demonstrated that rats exposed to continuous light have significantly higher levels of lipid per-oxidation in kidney (Mou *et al*., 2022). The finding also demonstrated that exposure to irradiation also increases the LPO level in relative spleen. Malondialdehyde (MDA) level was highly significant ($P \leq 0.001$) in kidney and spleen as compared to control rats (Baydas *et al.,* 2001). TBARS level was increased because of excessive free radical load (Rai *et al*., 2016). H_2O_2 is an important ROS produced by incomplete reduction of molecular oxygen in the mitochondrial electron transport chain and is a byproduct of mitochondrial respiration. A significant rise in oxidative stress brought on by continuous light exposure in experimental rat kidney and spleen as compared to control are in conformity with the earlier reports (Yoshida *et al*., 2015). This oxidative imbalance was not visible only in the excessive generation of free radicals but also in a considerable decrease in the activity of antioxidant enzymes overall (Tunez *et al.,* 2003).Moreover, exogenous administration of melatonin reduces the MDA level in kidney and spleen directly by scavenging the free radicals (Rai *et al*., 2009; Zaharan *et al*., 2015). Our observation supports the earlier report of Moreno *et al*. (2005) and Vinogradova *et al*. (2010) that deprivation of nocturnal melatonin by constant light exposure (physiological pinealectomy) affected the level of antioxidant enzymes activities. A significant decreased level of superoxide dismutase (SOD), Catalase (CAT), and Reduced glutathione (GSH) was noted in kidney and spleen of the rat following photoperiodic stress. Exogenous administration of melatonin increases the level of reduce GSH by neutralizing the free radicals being in electron rich molecule (Younis *et al*., 2019), and also significantly increases the level of other antioxidant enzymes indirectly by activating their enzymatic expression.

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