

## Acutely applied melatonin prevents CCl<sub>4</sub>-induced testicular lesions in rats: the involvement of the oxidative capacity and arginine metabolism

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Carbon tetrachloride (CCl<sub>4</sub>) represents an organic chemical that causes reactive oxygen species derived organ disturbances including male infertility. Melatonin (MLT) is a neurohormone with strong antioxidant capacity, involved in numerous physiological processes. In this study we evaluated the capability of MLT, administered in a single dose of 50 mg/kg, to preserve the testicular tissue function after an acute administration of CCl<sub>4</sub> to rats. The disturbance in testicular tissue and the effects of MLT after CCl<sub>4</sub> exposure were estimated using biochemical parameters that enabled us to determine the tissue (anti)oxidant status and the intensity of arginine/nitric oxide metabolism. Also, the serum levels of testosterone and the histopathological analysis of tissue gave us a better insight into the occurring changes. A significant diminution in tissue antioxidant defences, arginase activity and serum testosterone levels, followed by the increased production of nitric oxide and extensive lipid and protein oxidative damage, was observed in the CCl<sub>4</sub>-treated group. The application of MLT after the CCl<sub>4</sub> caused changes, clearly visible at both biochemical and histological level, which could be interpreted mainly as a consequence of general antioxidant system stimulation and a radical scavenger. On the other hand, the application of MLT exerted a limited action on the nitric oxide signalling pathway.

**Keywords:** Testis. Melatonin. Carbon tetrachloride. Oxidative damage. Arginine metabolism.

### INTRODUCTION

Although there are numerous legal regulations related to the carbon tetrachloride (CCl<sub>4</sub>) management, this organic industrial compound still remains a major hazard risk to the ones exposed (Sherry *et al.*, 2018). This industrial pollutant, which can enter the organism through various routes, is known to cause multi-organ damage by altering cell/tissue function and structure (Sönmez *et al.*, 2014). When CCl<sub>4</sub>

comes in contact with the organism it is rapidly metabolized in all tissues that possess cytochrome P-450 enzymes (Slater, Cheeseman, Ingold, 1985). The products of this metabolism are highly reactive trichloromethyl (CCl<sub>3</sub>·) and trichloromethyl peroxy (CCl<sub>3</sub>O<sub>2</sub>·) radicals that are the main culprits of cell macromolecule damage (Radulović *et al.*, 2013; Wu, Zhang, Song, 2018). Among the cell components most vulnerable to the damaging potential of these generated free radicals are polyunsaturated fatty acids, which represent major building molecules of cell and organelle membranes (Manibusan, Odin, Eastmond, 2007).

A decline in male fertility can be the consequence of the exposure to numerous exogenous agents such as

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smoking, alcoholism, drugs (prescribed and non-prescribed) and even some toxic chemicals (e.g.  $\text{CCl}_4$ ) (Türk *et al.*, 2016). Cell membrane fluidity, depending on the amounts of polyunsaturated fatty acids, is suggested to be needed for the adequate functioning of spermatozoa and germ cells. Having this mind, one can say that these cells are highly susceptible to the reactive oxygen species (ROS) damaging potential (Khan, 2012; Türk *et al.*, 2016). Since the organs that are part of the male reproductive system express cytochrome P-450, it is not unexpected that they could suffer from exposure to  $\text{CCl}_4$  either (Türk *et al.*, 2016). Apart from ROS, the formed nitric oxide (NO), from arginine in the presence of inducible nitric oxide synthase (iNOS), is known to react with  $\text{O}_2^-$  thus generating highly reactive peroxynitrites that could damage biomolecules to a similar manner as ROS (Abdel Moneim, 2016).

Melatonin (MLT) is a neurohormone produced primarily by the pineal gland cells, however, contemporary studies suggest that it might be synthesized by numerous other cells (Sokolović *et al.*, 2018). The main role of MLT is passing the information about the daily cycle of light and darkness to the body, additionally, it regulates core temperature, sleep-wake rhythms, immune system function, tissue antioxidative defences, hemostasis and glucose levels (Claustrat, Brun, Chazot, 2005). Several studies dealt with MLT's potential in preventing testicular tissue damage in different animal models (Parlaktas *et al.*, 2008; Mirhoseini *et al.*, 2014; Sokolović *et al.*, 2015; El-Shafaei *et al.*, 2018), and there is only one report dealing with the effects of pre-treatment with MLT on acute testis damage induced by  $\text{CCl}_4$  (Wang *et al.*, 2018).

The aim of our study was to evaluate whether the exogenous MLT administered after  $\text{CCl}_4$  would prevent rat testicular tissue damage by determining the changes in serum and tissue biochemical parameters, as well as in tissue morphology.

## MATERIAL AND METHODS

### Chemicals, drugs, reagents, and instruments

All solvents (analytical grade) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), while commercial ELISA kits were obtained from AbCam

(Cambridge, UK). Melatonin (>95%) was dissolved in absolute ethanol and further diluted in a sterile saline solution (0.85% NaCl) prior to the administration at a dose of 50 mg/kg. The dose, treatment regimen and route of application (intraperitoneal injection; i.p.) were chosen based on the previous publications (Sokolović *et al.*, 2018; Ničković *et al.*, 2018). In these publications the efficacy of MLT in preventing brain ischemic and traumatic injury, liver and pancreatic damage was proven (Ničković *et al.*, 2018 and references cited within). Spectrophotometric analyses were performed using V-1800 Shimadzu spectrophotometer and/or microplate reader (Multiscan Ascent; Labsystems, Helsinki, Finland).

### Animals and housing

Healthy male Wistar rats (250–300 g, n=24) were obtained from the Vivarium of the Institute of Biomedical Research, Medical Faculty, Niš, Serbia. The animals were maintained under standard husbandry conditions in plastic cages with a temperature of  $23 \pm 2$  °C, relative humidity  $55 \pm 10\%$  and 12/12 h light/dark cycle. All animals were fed with standard laboratory food pellets, and water was provided *ad libitum*. The experiments were performed in accordance with the Declaration of Helsinki and European Community guidelines for the ethical handling of laboratory animals (EU Directive of 2010; 2010/63/ EU). The experimental procedures were commenced after being approved by the Animal Ethics Committee of the Republic of Serbia. The ethical approval number is 323-07-01762/2019-05, and the approval was obtained on 1<sup>st</sup> February, 2019.

### Experimental design

Before the experiment, animals were divided into four equal experimental groups (6 animals per group) and further housed in large spacious cages throughout the experiment. The acute testicular injury was induced by injecting a single dose of  $\text{CCl}_4$  (50%, v/v). The groups of animals were treated as follows:

Group I (Vehicle control): Animals treated with vehicle (0.85% NaCl saline solution containing 8% ethyl alcohol)

once 24 hours before the end of the experiment in a volume of 1 ml/kg.

Group II (MLT control): Animals treated once with MLT (50 mg/kg) in a volume of 1 ml/kg.

Group III (CCl<sub>4</sub> control): Animals were given a single CCl<sub>4</sub> dose (1 ml/kg).

Group IV (CCl<sub>4</sub> + MLT): Animals treated with MLT (50 mg/kg) once 1 h after a single CCl<sub>4</sub> dose.

All animals were sacrificed by an overdose of ketamine (Ketamidol, 10%) 24 hours after the last treatment and a blood sample was withdrawn by a cardiac puncture using a vacutainer system. One testis was collected for homogenization and further biochemical analyses (stored at -80 °C), while the second one was fixed in 10% formalin and processed following routine pathohistological techniques.

## Biochemical analyses

### *Serum isolation and determination of testosterone levels*

After blood collection, the tubes were left to clot at room temperature and were centrifuged at 3000 rpm for 10 min in order to obtain serum which was used for testosterone levels determination. Serum testosterone levels were determined using a standard Rat Testosterone ELISA kit (Cusabio Biotech. Co., LTD, Wuhan, Hubei, China), where the detection limits were from 0.13 ng/mL to 25.6 ng/mL, with an assay sensitivity of 0.06 ng/ml. The procedure was performed following the manufacturer's instructions.

### **Tissue homogenization and biochemical parameter determination**

Testicular tissue was homogenized in an ice-cold distilled water (10%, w/v) and the obtained homogenate was centrifuged at 12,000 rpm, for 10 min at 4°C in order to obtain a clear supernatant which was further used for the determination of tissue biochemical parameters. The protein content in supernatants was determined by using Lowry's method (Lowry *et al.*, 1951) and the amount

of proteins was calculated based on the bovine serum albumin standard curve.

## Determination of enzymatic antioxidants

Superoxide dismutase activity in testicular tissue homogenates was determined using colorimetric SOD determination kit (Abcam653454) following the manufacturer's instructions. The assay is based on the reaction of water-soluble tetrazolium salt (WST) as a reagent, which in the presence of superoxide anion results in the decrease of WST formation that can be detected at 450 nm. The obtained results are presented as the percent inhibition of the superoxide anions compared to the control group.

Glutathione peroxidase (GPx) activity was determined in rat testes homogenates using a standard spectrophotometric method that is described elsewhere (Sokolović *et al.*, 2018). The method is based on the reaction between the GSH, which remains after the enzymatic reaction that utilizes GSH and H<sub>2</sub>O<sub>2</sub>, and DTNB reagent. The enzyme activity was expressed as U/mg of proteins.

The catalase activity was measured spectrophotometrically, after the reaction of the enzyme with H<sub>2</sub>O<sub>2</sub> as a substrate and ammonium molybdate, at 405 nm. The reaction mixture consisted of tissue homogenate and reaction buffer and was initiated by H<sub>2</sub>O<sub>2</sub> (Ničković *et al.*, 2018). After incubation at room temperature for 1 min, the reaction was stopped by adding ammonium molybdate solution. The activity was expressed as CAT U/g of testicular tissue proteins.

## Determination of total tissue antioxidant capacity

Testes tissue antioxidant capacity (TAC) was determined by using a colorimetric assay kit (Abcam65329). The reaction was based on the conversion of Cu<sup>2+</sup> ions to Cu<sup>+</sup> ions. The degree of conversion was measured calorimetrically at 570 nm. The obtained results are expressed as a change in % activity from the one obtained in testicular tissue of the untreated animals.

### **Determination of non-enzymatic antioxidant capacities**

The amount of GSH in testes tissue homogenates was determined using standard Ellman method, which is based on the reaction of non-protein thiols with DTNB reagent. The absorbance of the reaction mixture was measured at 412 nm, where the amounts of GSH were determined using a standard curve constructed with different concentrations of GSH. The obtained results are expressed as  $\mu\text{mol}$  of GSH/mg of proteins.

### **Determination of G6PD activity**

The activity of G6PD was determined using a commercial colorimetric kit (Abcam, USA; ab102529). The reaction was based on a decrease in NADH concentration in the presence of the G6PD substrate. The enzyme activity was calculated based on the standard curve values obtained using a standard enzyme solution. The results are expressed as mU/g of tissue proteins.

### **Determination of biochemical parameters related to arginine metabolism**

The arginase activity was determined using a colorimetric method which is based on a reaction between ornithine and ninhydrin. The coloured reaction absorbance was measured at 515 nm and the obtained values were corrected with those obtained from the control (Porembaska, Kedra, 1975). The results are expressed as nmol/mg of proteins.

The quantitative determination of rat testis tissue iNOS was performed using CUSBIO (CSB-08325r) sandwich enzyme immunoassay kit following the manufacturer's instructions. The obtained values are expressed as U/mg of tissue proteins.

The concentration of nitrates/nitrites present in testicular tissue homogenates was determined in a mixture consisting of tissue homogenate and Griess reagent (Radulović *et al.*, 2015). The absorbance of the mixture was measured at 540 nm using a microplate reader and the final concentrations were calculated using a standard curve of sodium nitrate. The final concentration

of nitrates/nitrites in the sample was expressed as nmol/mg of testicular tissue proteins.

The amount of citrulline in testis tissue homogenate was determined according to a standard method that is based on a reaction with diacetyl monoxime and thiosemicarbazide (Boyde, Rahmatullah, 1980). The absorbance of the developed colour was measured at 530 nm and the results were presented as nmol/mg of proteins.

### **Lipid and protein damage determination**

The amount of lipid peroxidation was measured using a spectrophotometric in which the malondialdehyde (MDA) reacts with thiobarbituric acid, under increased heat, and forms a coloured product that can be measured at 532 nm (Sokolović *et al.*, 2018). The concentration of MDA in testicular tissue was calculated using a standard curve and the results are expressed as nmol of MDA per mg of tissue proteins.

Protein carbonyl content was used for the quantification of oxidatively modified proteins. Their content was determined spectrophotometrically using 2,4-dinitrophenylhydrazine as a colour reagent (Levine *et al.*, 1994). Reactive carbonyl derivatives were assessed using a molar extinction coefficient for 2,4-dinitrophenylhydrazine at 370 nm ( $22 \times 10^3$  L/mol/cm) and the obtained results are expressed as  $\mu\text{mol/g}$  of testicular tissue proteins.

### **Pathological analysis**

The fixed testis was processed using routine histological techniques that include tissue dehydrated through graded concentrations of ethyl alcohol and further embedment in paraffin wax. Tissue was cut into  $4\mu\text{m}$  thin slices and stained with Mayer's haematoxylin and eosin (HE). The changes in the structure of the testicular tissue were scored following a previously described Johnsen scoring system (Johnsen, 1970). From each animal, a randomly selected testis was fixated and examined. The score was based on the analysis of at least 20 tubules from each tissue section and the results are given as a mean value for the selected group. In each of the seminiferous

tubule, the level of sperm maturation and the germ cell appearance and their presence was examined in order to obtain a score value for a single tubule.

### Statistical analysis

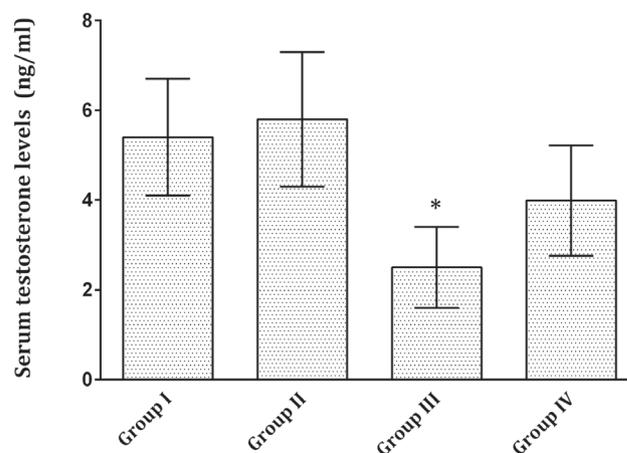
The obtained results are presented as mean values  $\pm$  standard deviations (SD). Statistically significant differences were determined by One-Way Analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons (GraphPad Prism, ver. 5.03; San Diego, CA). Probability values (p) less than, or equal to, 0.05 were considered to be statistically significant.

## RESULTS AND DISCUSSION

The disturbance in testicular tissue antioxidant/oxidant capacities is known to lead to male infertility, since a decline in antioxidant capacities leads to poor sperm quality and function (Wright, Milne, Leeson, 2014). By applying MLT to animals exposed to toxicant inducing testes tissue oxidative damage, this study was designed to determine whether this neurohormone could be used for potential treatment of infertility or at least in part to prevent testicular tissue damage. The application of CCl<sub>4</sub> has been previously proven to decrease male rat reproductive organ weight (testes, epididymis, and accessory sex organs) (Sönmez *et al.*, 2014), which was corroborated by the results of this study as well. The obtained relative for testis weight indicates that the exposure to CCl<sub>4</sub> statistically significantly decreased tissue weight, compared to the control group. The application of MLT after CCl<sub>4</sub> did not cause a significant amelioration in testis tissue weight, however, the value was not significantly different from the one obtained for the control group either (data not shown).

Different factors, such as testosterone levels, are previously found to be related to a decrease in male reproductive organ weight (Sönmez *et al.*, 2014), where the disturbance in androgenesis causes negative changes to testicular tissue mass (Klinefelter and Hess, 1998). The serum testosterone levels in rats exposed to CCl<sub>4</sub> were significantly decreased compared to both untreated animals (group I) and to those treated with MLT only

(group II) (Figure 1). The acute treatment with MLT after exposure to CCl<sub>4</sub> (group IV) was not able to significantly prevent a decrease in the serum testosterone levels, but the testosterone levels in this group were not different from the ones measured in the serum of animals from the control group (Figure 1).



**FIGURE 1** - Serum testosterone levels obtained from rats from different experimental groups. Data are shown as mean  $\pm$  SD (n=6). \*p<0.001 vs. Control (group I).

Such results related to a decrease in the serum testosterone levels in the rats treated with CCl<sub>4</sub> are in agreement with a slight decrease in testes tissue mass (Figure 1). Here again, the application of MLT did not prevent, at least not significantly, a decrease in testosterone concentrations, although the effect is notable since the values are not different from the ones found in the control group of animals. These results are in line with the described physiological functions of MLT. Melatonin directly inhibits, at the level of hypothalamus and hypophysis, the release of gonadotropin-releasing hormone and luteinizing hormone (Li, Zhou, 2015). Thus, it is unlikely that MLT can increase testosterone levels by acting at the hypothalamo-hypophyseal axis. The maintenance of testosterone levels is probably related to the MLT ability to preserve Leydig cells viability/function that was challenged by the CCl<sub>4</sub> metabolites and/or excessively produced ROS.

Cell antioxidant potential is tightly regulated by both enzymatic and non-enzymatic antioxidant systems and any disturbance in these precisely regulated systems,

the one that could be seen after CCl<sub>4</sub> application, leads to the disturbance in normal cell function (Bin-Jaliah, Sakr, 2018). In the present study, we determined the activity of different enzymes (SOD, CAT, GPx, and G6PD) and non-enzymatic antioxidant (GSH), whose function is related to the elimination of harmful ROS, and found them to be statistically significantly decreased, compared to the control group, after the exposure to CCl<sub>4</sub> (group III) (Table I). The two most prominent enzymes involved in the removal of harmful ROS are CAT and SOD. These two enzymes are found to be significantly decreased in the rats exposed to CCl<sub>4</sub>, possibly due to the significant enzyme activation in order to eliminate ROS or due to the enzyme activity inhibition in the presence of ROS

(Ighodaroa, Akinloye, 2018). When MLT was applied to the healthy animals, group II, it did not cause any significant changes in the enzymatic and non-enzymatic antioxidant capacities of rat testicles compared to group I (Table I). The application of MLT after exposure to CCl<sub>4</sub> led to the preservation/generation of both enzymatic and non-enzymatic antioxidant defences in testicular tissue, however, in the case of SOD, CAT and GPx activity, and in the case of TAC concentrations the effect of MLT was found to be significant (Table I). The observed activity of MLT might be related to the ability of this neurohormone to increase the expression of these enzymes, as well as to its ability to scavenge free radicals (Sokolović *et al.*, 2018).

**TABLE I** - Antioxidant capacities, and arginine-nitric oxide metabolism parameters estimated in testicular tissue obtained from rats belonging to different experimental groups

Parameter/Group	I (Vehicle control)	II (MLT control)	III (CCl <sub>4</sub> control)	IV (CCl <sub>4</sub> + MLT)
<b>Enzymatic and nonezymatic antioxidants</b>				
SOD (% of decrease in activity)	100±5	106.6±5.7	71.5±7.2*	98.6 ± 11.5 <sup>#</sup>
GPx (U/mg of proteins)	1.15±0.06	1.10±0.07	0.94±0.07*	1.09±0.04 <sup>#</sup>
CAT (U/g of proteins)	52.1±1.9	60.3±8.9	45.7±3.2***	59.6±12.4 <sup>##</sup>
G6PD (mU/g of proteins)	70.9± 3.3	66.6±4.1	51.5±0.7*	59.1±11.2***
GSH (nmol/g of proteins)	51.2±1.8	49.6±1.9	40.3±1.7*	42.6±1.3*
TAC (mM)	59.7±0.3	60.9±2.1	52.5±0.5*	55.8 ±0.4*, <sup>#</sup>
<b>Arginine-nitric oxide metabolism</b>				
NO (nmol/mg of proteins)	46. ±2.0	45.7±2.4	50.5±1.2**	48.3±1.5**
Citrulline (nmol/mg of proteins)	82.5±5.8	79.8±10.2	320.2±129.5 <sup>#</sup>	110.6±60.8 <sup>#</sup>
Arginase activity (nmol/mg of proteins)	118.9±56.4	92.6±29.6	2.5±5.3*	7.3±3.2*
iNOS activity (U/mg of proteins)	29.5±4.6	17.7±5.6***	90.7±9.9*	81.2±3.2*

Values are given as means values ± SD, n=6. One-way ANOVA followed by Tukey’s test was used to compare the groups. \*p<0.001; \*\*p<0.01; \*\*\*p<0.05 vs. Control (group I), <sup>#</sup>p<0.001; <sup>###</sup>p<0.05 vs. CCl<sub>4</sub> treated animals (group III).

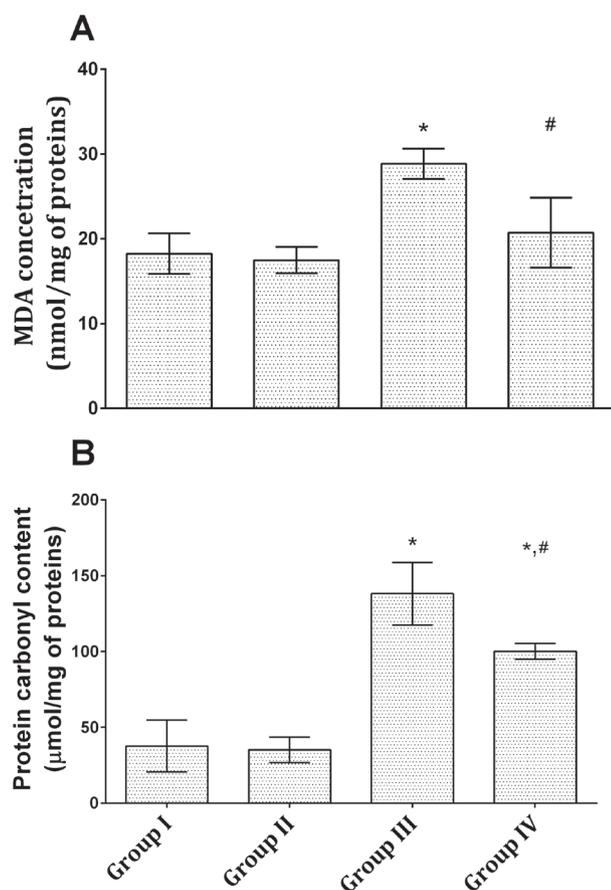
**TABLE II** - Johnsen's testicular score estimated for each experimental group

Group	I (Vehicle control)	II (MLT control)	III (CCl <sub>4</sub> control)	IV (CCl <sub>4</sub> + MLT)
Johnsen's testicular score	10	10	8.5	9

The enzymes that utilize GSH as a substrate for toxic products elimination, such as GPx, can be considered useful parameters in biomonitoring tissue oxidative capacities (Song *et al.*, 2013). The activity of GPx, as well as the concentrations of GSH, in testicular tissue of rats exposed to CCl<sub>4</sub>, were found to be decreased (Table I), probably due to their excessive "consumption" in order to eliminate toxic ROS. Although MLT was able to partially preserve tissue GPx activity, the concentration of GSH still remained decreased compared to the untreated control (Table I). Additionally, the enzyme whose activity was found to be decreased by the exposure to CCl<sub>4</sub> is G6PD (Table I). The G6PD deficiency is connected to the increased sensibility of cells towards different ROS, as well as to the action of NO and peroxynitrite (Ho, Cheng, Chiu, 2007). The application of MLT was previously reported to increase the activity of G6PD in both isolated erythrocytes (*in vitro*) and in rats a few hours (1-4 h) after its intraperitoneal injection (Ho, Cheng, Chiu, 2007). In a similar manner, although not absolute, we found that the application of MLT prevented a decline in testicular G6PD activity induced by CCl<sub>4</sub> application. Thus, this kind of activity could be brought in connection with MLT possible therapeutic potential for G6PD deficiency anemias (Ciftçi, Bilici, Küfrevioğlu, 2001) and yet again to its ability to increase tissue antioxidant capacities by increasing the intracellular (cytosolic) pool of NADPH (Ho, Cheng, Chiu, 2007). Determination of TAC reflects all substances (small molecules and proteins) that are involved in the protection of tissue from excessive ROS production (Sokolović *et al.*, 2018). As expected, a decrease in TAC was found in the animals exposed only to CCl<sub>4</sub>, probably due to the significant reaction of

antioxidant with ROS of different origin. When applied on its own, MLT did not alter TAC, which is not completely in accordance with some previous reports where MLT caused an increase in plasma TAC (Grzelak *et al.*, 2005). However, when MLT was given to animals after CCl<sub>4</sub>, the concentration of TAC was increased (Table I). Such discrepancy might be related to MLT ability to affect TAC only when there is significant ROS production, but not under normal (physiological) ROS production.

As explained in the introductory part of this study, an increase in ROS causes polyunsaturated fatty acids damage, thus initiating a chain reaction which potentially could alter spermatozoa cell membrane fluidity (Khan, 2012; Türk *et al.*, 2016). Oxidatively modified lipids, estimated through the levels of MDA, were found statistically significantly increased in the animals exposed to CCl<sub>4</sub>, compared to the untreated ones (Figure 2A). The treatment with MLT was able to completely prevent an increase in MDA that follow CCl<sub>4</sub> application, maintaining the levels of MDA at the concentrations similar to those found in healthy animals (Figure 2A). An increase in MDA content was found to negatively correlate with sperm count, motility and morphology in men (Benedetti *et al.*, 2012). Although this is the first study evaluating MLT's potential in preventing testicular tissue damage induced by CCl<sub>4</sub>, it does not come as a surprise that MLT prevented an increase in MDA content. These results could partially be expected since some of the previous studies provided us with evidence that this neurohormone inhibits testicular tissue MDA formation induced by different toxic substances (Parlaktas *et al.*, 2008; Mirhoseini *et al.*, 2014; Sokolović *et al.*, 2015; El-Shafaei *et al.*, 2018).

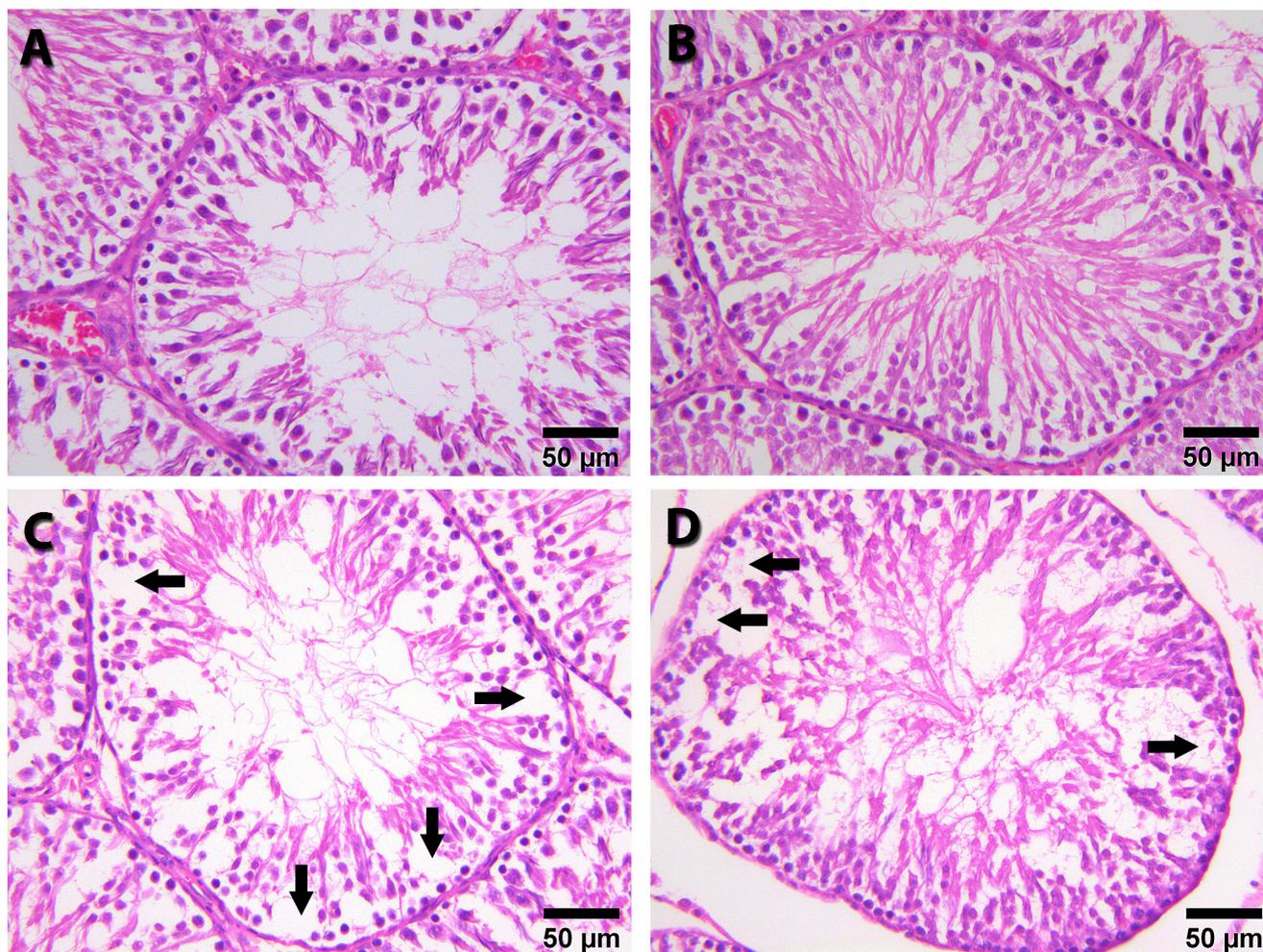


**FIGURE 2** - Testicular tissue MDA (A) and carbonylated protein (B) concentrations obtained from different experimental groups. Data are presented as mean  $\pm$  SD (n=6). \*p<0.001 vs. Control (group I), #p<0.001 vs. CCl<sub>4</sub> treated animals (group III).

Apart from lipids, generated ROS could oxidatively modify amino acids i.e. proteins, where a protein carbonylation is considered an early biomarker of oxidative tissue damage (Ong *et al.*, 2002). The amount of carbonylated protein content was significantly increased in the groups of animals that received CCl<sub>4</sub>, compared to the untreated ones (Figure 2B). A single dose of MLT

significantly prevented an increase in carbonylated protein content compared to the CCl<sub>4</sub> treated group, however, the level of the measured parameter still remained higher than in the control group of animals (Figure 2B). Such protein modification is found to affect protease activity, thus causing a decrease in their activity and consequently their removal (Kumar *et al.*, 2007). The administration of MLT failed to completely prevent the formation of this biomarker to the same extent as it did in the case of MDA (Figure 2B). Previous studies also proved MLT's potential in preventing protein carbonylation process in testicular tissue of rats subjected to ischemia and reperfusion injury (Kurcer *et al.*, 2008) or exposed to different toxic substances (Parlaktas *et al.*, 2008), and radiation (Sokolović *et al.*, 2015).

The pathohistological analysis did not reveal any lesions in testicular tissue of the control (group I) and the melatonin-treated (group II) group (Figure 3A and B). In the groups that received CCl<sub>4</sub>, with or without MLT, microscopic analysis revealed occasional loss of germ cells compared to the untreated animals (Figure 3C and D). These oxidative lipid/protein changes in testicular tissue partially overlap with a mild reduction in germ cell number (Figure 2 and 3). This reduction should not be regarded as mild since a short exposure to CCl<sub>4</sub> (only 24 hours) can be regarded as an acute one, and the extensive changes are expected after chronic exposure (Türk *et al.*, 2016; Wang *et al.*, 2018). The single previous study proved that MLT in a dose of 10 mg/kg applied after CCl<sub>4</sub> can prevent germ cell damage and can cause a reduction in testicular cells depolarization. The authors suggested that the mechanism of MLT action potentially involves regulation of tissue antioxidative capacities and downregulation of telomerase and caspase-3 (Wang *et al.*, 2018).



**FIGURE 3** - Morphological appearance of testicular tissue obtained from different experimental groups, HE staining (x200 magnification). (A) and (B) - Normal testicular tissue with preserved seminiferous tubule structure and a normal number of sperms within the lumen observed in group I and II, respectively; (C) and (D) – Mild disturbance in seminiferous tubule structure with occasional loss of germ cells (arrow) seen in group III and IV, respectively.

The role of NO in the pathogenesis of the CCl<sub>4</sub> mediated testicular tissue damage has been previously evaluated, and a significant increase in NO concentrations and iNOS activity was detected in the animals treated with this toxic chemical (Abdel Moneim, 2016), which is the case in the present study as well (Table I). Also, one may say that the exposure to this toxicant caused a shift in arginine metabolism in testicular tissue, where this metabolism is changed from arginase (significantly decreased) to iNOS (significantly increased) (Table I). The iNOS activity and NO do not primarily originate from inflammatory cells, as is the case in some other tissues

(e.g. liver) since no significant increase in inflammatory cells has been observed on the examined tissue sections. Probably the source of the investigated arginine/nitric oxide metabolism parameters are Sertoli, Leydig and germ cells whose function has been previously reported to be dependent on this signalling pathway (Auharek *et al.*, 2012). Since the NO is found to be involved in testosterone production/secretion regulation (Jarazo *et al.*, 2015), it is interesting that MLT maintained testosterone levels, but did not affect (decreased) NO production (Table I). This can be probably again explained by some other mechanism of action of MLT

that affects cell function directly, rather than through other mediators. Besides the cell macromolecules damage induced by peroxy nitrates, the generated NO is found to promote  $\alpha$ -fodrin proteolysis in germ cells, consequently causing cell necrosis (Shiraishi, Naito, Yoshida, 2001). The findings of the present study are in line with such mechanism since the rare loss of germ cells was seen and the Johnsen's testicular score was almost identical in the two groups exposed to  $\text{CCl}_4$  (Table II), and the levels of NO were not different either (Table I).

In conclusion, melatonin was found to be effective in reducing carbon tetrachloride induced rat testicular toxicity by increasing tissue's enzymatic and non-enzymatic capacities and by preventing lipid and protein oxidative damage. However, it was found MLT had very little impact on the investigated arginine/nitric oxide metabolism parameters and on the limited changes in the microscopic testicular tissue induced by  $\text{CCl}_4$ . Our results suggest that MLT acts rather as a general antioxidant system stimulator and a radical scavenger than as a modulator of the nitric oxide signalling pathway.

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## REFERENCES

Abdel Moneim AE. Prevention of carbon tetrachloride ( $\text{CCl}_4$ )-induced toxicity in testes of rats treated with *Physalis peruviana* L. fruit. *Toxicol Ind Health*. 2016;32(6):1064-73.

Auharek SA, Lara NL, Avelar GF, Sharpe RM, França LR. Effects of inducible nitric oxide synthase (iNOS) deficiency in mice on Sertoli cell proliferation and perinatal testis development. *Int J Androl*. 2012;35(5):741-51.

Benedetti S, Tagliamonte MC, Catalani S, Primiterra M, Canestrari F, De Stefani S, et al. Differences in blood and semen oxidative status in fertile and infertile men, and their relationship with sperm quality. *Reprod Biomed Online*. 2012;25(3):300-306.

Boyde TR, Rahmatullah M. Optimization of conditions for the colorimetric determination of citrulline, using diacetyl monoxime. *Anal Biochem*. 1980;107(2):424-431.

Ciftçi M, Bilici D, Küfrevioğlu OI. Effects of melatonin on enzyme activities of glucose-6-phosphate dehydrogenase from human erythrocytes in vitro and from rat erythrocytes in vivo. *Pharmacol Res*. 2001;44(1):7-11.

Claustrat B, Brun J, Chazot G. The basic physiology and pathophysiology of melatonin. *Sleep Med Rev*. 2005;9(1):11-24.

Grzelak A, Bartosz G. Melatonin does not affect total antioxidant capacity of blood plasma in vitro. *Scand J Clin Lab Invest*. 2005;65(1):77-81.

El-Shafaei A, Abdelmaksoud R, Elshorbagy A, Zahran N, Elabd R. Protective effect of melatonin versus montelukast in cisplatin-induced seminiferous tubule damage in rats. *Andrologia*. 2018;50(9):e13077.

Ho HY, Cheng ML, Chiu DT. Glucose-6-phosphate dehydrogenase from oxidative stress to cellular functions and degenerative diseases. *Redox Rep*. 2007;12(3):109-18.

Ighodaroa OM, Akinloye OA. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria J Med*. 2018;54(4):287-93.

Jarazo Dietrich S, Fass MI, Jacobo PV, Sobarzo CM, Lustig L, Theas MS. Inhibition of NOS-NO system prevents autoimmune orchitis development in rats: relevance of NO released by testicular macrophages in germ cell apoptosis and testosterone secretion. *PLoS One*. 2015;10(6):e0128709.

Johnsen SG. Testicular biopsy score count--a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. *Hormones*. 1970;1(1):2-25.

Khan RA. Protective effects of *Launaea procumbens* on rat testis damage by  $\text{CCl}_4$ . *Lipids Health Dis*. 2012;11:103.

Klinefelter GR, Hess RA. Toxicity of the excurrent ducts and accessory organs in the male. In: Korach KS, editor. *Reproductive and Developmental Toxicology*, Marcel Dekker Inc; 1998. New York, p. 553-91.

Kumar TR, Muralidhara. Induction of oxidative stress by organic hydroperoxides in testis and epididymal sperm of rats in vivo. *J Androl*. 2007;28(1):77-85.

Kurcer Z, Oguz E, Ozbilge H, Baba F, Aksoy N, Celik N. Effect of melatonin on testicular ischemia/reperfusion injury in rats: is this effect related to the proinflammatory cytokines? *Fertil Steril*. 2008;89(5):1468-73.

Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol*. 1994;233:346-57.

- Li C, Zhou X. Melatonin and male reproduction. *Clin Chim Acta*. 2015;446:175-80.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem*. 1951;193(1):265-75.
- Manibusan MK, Odin M, Eastmond DA. Postulated carbon tetrachloride mode of action: a review. *J Environ Sci Health Part C*. 2007;25(3):185-209.
- Mirhoseini M, Saki G, Hemadi M, Khodadadi A, Mohammadi AJ. Melatonin and testicular damage in busulfan treated mice. *Iran Red Crescent Med J*. 2014;16(2):e14463.
- Ničković VP, Novaković T, Lazarević S, Šulović L, Živković Z, Živković J, et al. Pre- vs. post-treatment with melatonin in CCl<sub>4</sub>-induced liver damage: Oxidative stress inferred from biochemical and pathohistological studies. *Life Sci*. 2018;202:28-34.
- Parlaktas BS, Ozyurt B, Ozyurt H, Tunc AT, Akbas A. Levels of oxidative stress parameters and the protective effects of melatonin in psychosis model rat testis. *Asian J Androl*. 2008;10(2):259-65.
- Poremska Z, Kedra M. Early diagnosis of myocardial infarction by arginase activity determination. *Clin Chim Acta*. 1975;60(3):355-61.
- Radulović NS, Randjelović PJ, Stojanović N, Cakić ND, Bogdanović, G, Živanović AV. Aboriginal bush foods: a major phloroglucinol from Crimson Bottlebrush flowers (*Callistemon citrinus*, Myrtaceae) displays strong antinociceptive and antiinflammatory activity. *Food Res Int*. 2015;77(Pt 2):280-9.
- Radulović NS, Randjelović PJ, Stojanović NM, Ilić IR, Milojević BA. Influence of methyl and isopropyl *N*-methyl anthranilates on carbon tetrachloride-induced changes in rat liver morphology and function. *FU Phys Chem Tech*. 2013;11(1):67-73.
- Shiraishi K, Naito K, Yoshida K. Nitric oxide promotes germ cell necrosis in the delayed phase after experimental testicular torsion of rat. *Biol Reprod*. 2001;65(2):514-21.
- Sherry D, McCulloch A, Liang Q, Reimann S, Newman PA. Current sources of carbon tetrachloride (CCl<sub>4</sub>) in our atmosphere. *Environ Res Lett*. 2018;13:024004.
- Slater TF, Cheeseman KH, Ingold KU. Carbon tetrachloride toxicity as a model for studying free-radical mediated liver injury. *Philos Trans R Soc Lond B Biol Sci*. 1985;311(1152):633-45.
- Sokolović DT, Lilić L, Milenković V, Stefanović R, Ilić TP, Mekić B, et al. Effects of melatonin on oxidative stress parameters and pathohistological changes in rat skeletal muscle tissue following carbon tetrachloride application. *Saudi Pharm J*. 2018;26(7):1044-50.
- Sokolović D, Djordjevic B, Kocic G, Stoimenov TJ, Stanojkovic Z, Sokolovic DM, et al. The effects of melatonin on oxidative stress parameters and DNA fragmentation in testicular tissue of rats exposed to microwave radiation. *Adv Clin Exp Med*. 2015;24(3):429-36.
- Song J, Zhao M, Liu X, Zhu Y, Hu X, Chen F. Protection of cyanidin-3-glucoside against oxidative stress induced by acrylamide in human MDA-MB-231 cells. *Food Chem Toxicol*. 2013;58:306-10.
- Sönmez M, Türk G, Çeribaşı S, Çiftçi M, Yüce A, Güvenç M, et al. Quercetin attenuates carbon tetrachloride-induced testicular damage in rats. *Andrologia*. 2014;46(8):848-58.
- Türk G, Çeribaşı S, Sönmez M, Çiftçi M, Yüce A, Güvenç M, et al. Ameliorating effect of pomegranate juice consumption on carbon tetrachloride-induced sperm damages, lipid peroxidation, and testicular apoptosis. *Toxicol Ind Health*. 2016;32(1):126-37.
- Wright C, Milne S, Leeson H. Sperm DNA damage caused by oxidative stress: Modifiable clinical, lifestyle and nutritional factors in male infertility. *Reprod Biomed Online*. 2014;28(6):684-703.
- Wang TY, Li Y, Wu X, Yang X.L, Wang Y, Wang D. Protective effects of melatonin on CCl<sub>4</sub>-induced acute liver damage and testicular toxicity in rats. *Ind J Pharm Sci*. 2018;80(6):1100-07.
- Wu T, Zhang Q, Song H. Swertiamarin attenuates carbon tetrachloride (CCl<sub>4</sub>)-induced liver injury and inflammation in rats by regulating the TLR4 signaling pathway. *Braz J Pharm Sci*. 2018;54(4):e17449

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