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Evidence of melatonin ameliorative effects on the blood-testis barrier and sperm quality alterations induced by cadmium in the rat testis

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ABSTRACT

Herein, we further document the protective action of melatonin (MLT) in mitigating cadmium (Cd) effects on adult rat testis. Cd treatment provoked testicular injury, that was documented by histological and biomolecular alterations, i.e., decrease of serum and testicular testosterone concentration and modified sperm parameters. Mainly, both the cytoarchitecture of the blood-testis barrier (BTB) and germ cell morphology were perturbed, as highlighted by impairment in structural (OCN, VANGL, Cx43) and regulative (Src and FAK) protein levels and/or activation. The study focused on the involvement of the autophagy pathway, that was enhanced especially in the Sertoli cells, probably in response to the disorganization of the BTB. Results obtained with the MLT co-treatment demonstrated that its administration decreased the level of oxidative damage caused by Cd, with reversal of all the observed changes. Moreover, the beneficial effects of MLT alone were evidenced by an increase of sperm quality, in term of motility and DNA integrity. The combined results, obtained in rat, strongly encourage to consider a role for MLT in improving also human testicular health, not only in men exposed to Cd, but also in those having fertility disorders, to ameliorate sperm quality and, consequently, reproductive success.

1. Introduction

Infertility is one of the global public health concerns as it affects 15–20% of couples worldwide and, in males, it may be largely characterized by a reduced sperm (SPZ) quantity and quality (Bui et al., 2018). Growing evidence indicates that environmental pollutants contribute to its increasing frequency (Gabrielsen and Tanrikut, 2016). Among them, heavy metals have been directly linked to male infertility (Wirth and Mijal, 2010) and cadmium (Cd) is attracting strong attention due to its intrinsic toxicity and ability to modify the normal hormonal status, acting as an endocrine disruptor (Bhardwaj et al., 2021).

Cd is required in numerous industrial procedures, as anticorrosive agent, in the manufacture of nickel-cadmium batteries and phosphate fertilizers. So, not only the people working with Cd are directly and consciously exposed, but also the general population, mainly through the food chain, and especially by tobacco smoking (Wang et al., 2021a). With regard to other pollutants, Cd half-life is very long (20–40 years), leading to its bioaccumulation in human tissues, mostly in kidneys, bones, and gonads (Wang et al., 2021a).

It is well known that testis is highly sensitive to Cd toxicity which produces structural damage to the cells of both germinal and interstitial compartments, at various levels, and acting on germ cell (GC) proliferation and differentiation, blood-testis barrier (BTB) and vascular endothelium integrity (de Angelis et al., 2017; Zhu et al., 2020). Many studies reported that testicular Cd toxicity is the result of interactions of a complex network of causes, including impaired steroidogenesis, induced oxidative stress, autophagy, apoptosis and necrosis of GC, along with alterations in SPZ quality, which ultimately leads to a reduced fertility

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(Zhao et al., 2017). It is important to note, however, that autophagy is a key adaptive process occurring in response to stress induction or depletion of survival factors, reducing the accumulation of damaged molecules/organelles to prevent apoptosis (Mizushima et al., 2008).

The BTB is one of the main targets of Cd (de Angelis et al., 2017; Zhu et al., 2020). The BTB, a structure with features distinct to the testis [made of tight, adherens and gap-junctions between Sertoli cells (SC)], creates two compartments, the basal and the apical (Mruk and Cheng, 2015). BTB integrity is fundamental to avoid the passage of toxic agents from the blood into the tubules, but also to create an immune-privileged environment (Fijak and Meinhardt, 2006). Most of the proteins composing the abovementioned junctions [e.g., Occludin (OCN), Zonula occludens-1 (ZO-1), connexin 43 (Cx43) and Van Gogh-like protein 2 (VANGL2)] are integral membrane proteins, whose intracellular domains recruit regulatory proteins [i.e., Focal adhesion kinase (FAK) and Proto-oncogene tyrosine-protein kinase Src (c-Src)], as well as adapter factors that anchor the entire complex to the cell cytoskeleton (Lie et al., 2013). BTB is an exceptionally dynamic structure, since the junctions are continuously "disrupted" and "created" to permit the transit of preleptotene spermatocytes (SPC) to the apical compartment via changes in adhesive function and in the phosphorylation status of membrane proteins and/or their adapters affecting the association to the cytoskeleton (Mruk and Cheng, 2015).

Considering that Cd exposure is essentially an unavoidable phenomenon, together with its consequences on the testis, many studies focused on research aimed to identify the precise molecular targets of Cd toxicity and, subsequently, to find substances that may ameliorate or eliminate its toxic effects to be used for new therapeutic approaches.

Among these protective molecules, melatonin (MLT) is one of the most studied, since its antiapoptotic and antioxidant properties with the ability to remove oxygen and nitrogen-based destructive species are well documented (Kopustinskiene and Bernatoniene, 2021). In fact, we recently demonstrated that MLT protects rat bones and ovaries from Cd-induced toxicity, acting on different signaling pathways (Kechiche et al., 2021; Knani et al., 2020).

Taking in account that very few papers reported the protective effects of MLT against the Cd-induced toxicity in mammalian testis, and that they are exclusively focused on oxidative stress parameters (Eybl et al., 2006; Ji et al., 2012; Kara et al., 2007; Karbownik et al., 2001; Li et al., 2016), we recently added new insight in this field, demonstrating the efficacy of MLT in counteracting the Cd reprotoxicity-induced decrease in the cytoskeleton-related proteins DAAM1 and PREP (Venditti et al., 2021a). Herein, we further characterized the adverse consequence of Cd treatment and the putative mitigating action exerted by MLT on rat testicular physiopathology, with a special attention on serum and testicular testosterone (T) levels, autophagy (LC3B and p62), changes in the BTB (OCN, Cx43, VANGL2, Src, FAK) markers, other than on SPZ parameters and quality.

2. Materials and methods

2.1. Animals and experimental design

Twenty-four *Wistar* male rats, aged 2 months and weighting 225 ± 36 g, were kept in individual stainless-steel cages under controlled conditions of light (12:12 h light/dark), temperature (22 ± 2 °C) and humidity (55 ± 20%). Food and water were given ad libitum. Rats were randomly divided into four groups (n = 6 each): (1) control; (2) Cd-treated (50 mg $CdCl_2$/L in drinking water; Sigma-Aldrich); (3) MLT-treated (3 mg/L in drinking water; Sigma-Aldrich); (4) Cd + MLT-treated (50 mg $CdCl_2$/L + 3 mg MLT/L in drinking water). The MLT stock solution was prepared in ethanol; final ethanol concentration in drinking water was 0.015%. Groups 1 and 2 received an equivalent amount of ethanol in drinking water. The concentration of Cd and MLT used in this study were chosen according to literature (Kechiche et al., 2021; Knani et al., 2020). To protect MLT from light, water bottles, changed twice weekly, were covered with aluminum sheet. Treatment lasted 40 days and rats were weighted every 5 days. The experimental procedure was approved by the Ethics Committee for Research in life science and health of the Higher Institute of Biotechnology of Monastir (CER-SVS/ISBM- protocol 022/2020) and was carried out accordingly to the UNESCO Recommendation Concerning Science and Scientific Research (1974, 2017).

2.2. Sample collection

At the end of treatment, blood was taken by a cardiac puncture in heparinized tubes, and the animals were anesthetized with chloral hydrate and sacrificed. Blood was centrifuged at 3500 rpm for 15 min at 4 °C, plasma was collected and stored at − 80 °C for T assay. The testes were removed and weighted; for each rat, right testis was immersed in 10% neutral buffered formalin for histological studies, while the left was kept at − 80 °C for biomolecular studies. To evaluate the spermatogenic efficiency, the daily sperm production (DSP) per gram of testis was evaluated at the end of the experiment. Epididymides were used to collect SPZ for the evaluation of sperm parameters.

2.3. Sperm parameters

To evaluate sperm concentration and motility, SPZ were obtained by cutting one epididymis in 1 mL of RPMI culture medium (R0883, Sigma Aldrich) pre-heated to 32 °C (Chemek et al., 2018) and the other was minced in 1 mL of 0.9% saline mixed with 1 mL 10% neutral buffer formalin for morphological study, according to Chemek et al. (2018). All the slides were observed under the light microscope (Axiostar plus Zeiss) at X40 magnification.

For the evaluation of sperm DNA integrity, acridine orange (AO) staining was performed on formalin fixed SPZ (Tejada et al., 1984). Stained slides were examined using a fluorescent microscope (Leica DM 5000 B+CTR 5000) with a UV lamp and saved with IM 1000 software.

A total of about 250 SPZ was examined on each slide, and each sperm parameter was expressed as a percentage.

DSP was determined according to Chemek et al. (2018) using the following two formulas:

$$\mathbf{Y = X/10 * 100 * 5 * 20 * 1000} \quad (1)$$

where Y is the number of spermatids (SPT) present in the homogenate, X is the number of SPT that are counted in Mallasez chambers, 10 is the number of observed squares in one reading, 100 is the number of total squares in the chamber, 5 is dilutions made with saline solution, 20 μL is homogenate for loading the chamber, 1000 is to convert microliter into milliliter.

$$\mathbf{DSP = Y/6.3} \quad (2)$$

where 6.3 indicates the number of days these SPT remained in the seminiferous epithelium (SE).

Two hundred sperms were analyzed per slide.

2.4. Serum and testicular T concentration

Serum T level was quantified using the testosterone ELISA kit (#DE1559; Demeditec Diagnostics GmbH). The detection limit for T was defined at 0.083 ng/mL and the optical density was read at 450 nm. An ELISA reader (Λayto, RT-2100C) calculated the concentration automatically. Testicular T concentration was assayed according to Kechiche et al. (2021) using a commercial kit (#ab108666, Abcam).

2.5. Thiobarbituric acid-reactive species (TBARS) levels

To measure lipid peroxidation products, sperm TBARS levels were assayed according to Lama et al. (2019). Results were expressed as

TBARS μM/μg of extracted protein. Each measurement was performed in triplicate.

2.6. Protein extraction and Western blotting (WB) analysis

Proteins were extract from testis in RIPA lysis buffer [0.1% SDS, 1% NP-40, 100 mM sodium orthovanadate, 0.5% sodium deoxycholate, in PBS supplemented with protease inhibitors (4 μg/μL leupeptin, chymostatin, aprotinin, pepstatin A, and PMSF)]. The homogenized samples were sonicated 3 times (20 Hz for 20 s each), placed on ice for 30 min, centrifuged at 13.000 rpm for 30 min at 4 °C; finally, the resulting supernatants were collected. Forty micrograms of the protein extracts were separated by 9% SDS–PAGE and transferred to Hybond-P polyvinylidene difluoride membranes (#GE10600023; Amersham Pharmacia Biotech, Buckinghamshire, UK) at 280 mA for 2.5 h at 4 °C. Filters were blocked with 5% skim milk in TBST (10 mM Tris–HCl pH 7.6, 150 mM NaCl, containing 0.25% Tween-20) for 2 h at RT. Then, they were incubated with primary antibodies overnight at 4 °C diluted in the blocking solution. After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody anti-mouse IgG or anti-rabbit IgG secondary antibody. Then, membranes were washed again three times in TBST and the immunocomplexes were detected using the enhanced chemiluminescence (ECL)-WB detection system (Venditti et al., 2018).

Proteins were extracted from SPZ and processed as above described to perform a WB analysis for PREP, a protein involved in sperm motility (Venditti and Minucci, 2019). For details concerning all the used antibodies, see Table S1. ImageJ software (version 1.53 g) was used to analyze all bands. WB was performed in triplicate.

2.7. Immunofluorescence (IF) analysis

For IF staining, testis sections and sperm (for PREP localization only) were permeabilized with PBS pH 7.4 containing 0.1% Triton-X-100 for 30 min was performed for the proteins not located on the plasma membrane (LC3B and PREP). Antigen retrieval was performed by putting slides in a pressure cooker for 3 min in 0.01 M citrate buffer (pH 6.0). Then, non-specific binding sites were blocked with PBS containing 5% BSA and normal goat serum diluted 1:5. Later, sections were incubated with primary antibodies overnight at 4 °C (for details, see Table S1). After three washes in PBS, the appropriate secondary antibody diluted 1:500 in the blocking mixture was added for 1 h at RT. Finally, the cell nuclei were stained with Vectashield + DAPI. The slides were observed and captured with the optical microscope (Leica DM 5000 B + CTR 5000) with UV lamp and saved with IM 1000 software (Venditt et al., 2019). Two different negative controls were performed: (1) by using rat isotype IgG (#I5006, Sigma-Aldrich; Milan, Italy), (2) by omitting the primary antibody.

2.8. Statistical analysis

Data were reported as mean ± standard error (SEM). Differences between the groups were considered statistically significant at $p < 0.05$. Analyses were performed using one-way ANOVA, Tukey's post hoc *t*-test was applied when appropriate with Prism 5.0, GraphPad Software (San Diego, CA, USA).

3. Results

3.1. Body and relative testis weights, serum/testicular T concentrations

There was no effect of Cd and/or MLT on rat body weight gain during the experimental period (Fig. S1). Contrarily, the testicular weight in Cd-treated group significantly decreased as compared to the control ($p < 0.05$). The co-treatment with Cd and MLT ($p < 0.05$) caused a significant increase of the testicular weight as compared to the Cd-treated group (Table S2).

Serum and testicular T concentrations significantly decreased in the Cd-treated group as compared to the control ($p < 0.001$). The treatment with both Cd and MLT counteracted the T reduction induced by Cd (Table S2).

3.2. Effect of Cd and/or MLT on autophagy

To verify whether autophagy is a mechanism activated by Cd toxicity, LC3B and p62, autophagy markers, were analyzed (Fig. 1). WB analysis showed that Cd treatment caused a significant increase of LC3B-II protein level as compared to the control ($p < 0.001$; Fig. 1A, B). However, the co-administration of MLT reduced LC3B-II protein level as compared to the Cd group ($p < 0.05$), but it was not completely reverted to the control level ($p < 0.01$).

p62 protein level showed an opposite behavior, since Cd treatment significantly decreased its level, as compared to the control ($p < 0.01$), confirming the increased rate of autophagy. MLT alleviated the increase of p62 level, since no difference was observed between control and Cd+MLT groups (Fig. 1A, C).

The activation of autophagy was further confirmed by LC3B IF staining, shown in Fig. 1D. The signal was specifically localized in the SC cytoplasm surrounding the GC (arrowheads) in all the groups, but more intense and extended in the Cd-treated animals.

3.3. Effect of Cd and/or MLT on BTB markers

Cd treatment produced consistent alterations in the BTB at both structural and regulatory proteins, as compared to all the other groups (Fig. 2). Cd exposure resulted in a significant reduction in the OCN ($p < 0.01$; Fig. 2A, B), VANGL2 ($p < 0.05$; Fig. 2A, C) and Cx43 ($p < 0.001$, Fig. 2A, D), as well as in the phosphorylation status of p-Src ($p < 0.01$; Fig. 2A, E) and p-FAK ($p < 0.01$; Fig. 5A, F) as compared to control. No difference between Cd+MLT and control was observed (Fig. 2A–F). Surprisingly, MLT alone induced a significant rise of protein levels of all the three structural markers as compared to the control ($p < 0.001$; Fig. 2A–D).

To characterize the effects exerted by Cd and/or MLT on OCN, VANGL2 and CX43 localization, an IF analysis was performed (Figs. 3 and 4). OCN, a tight junction integral protein composing the BTB, clearly localized in the SC cytoplasm (arrowheads; Fig. 3A; insets), both at the basal compartment and in the protrusions surrounding the GC, in all the groups; but a much lower intensity was observed in the testis of the Cd-treated group.

VANGL2, a protein belonging to the Planar Cell Polarity family, regulates the apical ectoplasmic specialization (ES) at the SC-SC and SC-SPT interface. In the testis of control, VANGL2 localized specifically in the SC cytoplasm (arrowheads; Fig. 3B; insets), and in the protrusions surrounding the SPT/SPZ heads (arrows; Fig. 3B), showing a clear striped conformation. In the Cd-treated group, a weaker signal and a general "disorganization" of VANGL2 distribution in the SC protrusions were observed (arrows; Fig. 3B). MLT co-treatment, ameliorated VANGL2 localization, since it was comparable to that observed in the control. Finally, a positive signal was detected in the Leydig cells (LC) of all the analyzed groups (asterisks; Fig. 3B).

Cx43 is the predominant testicular gap-junction protein, occurring between adjacent SC and at SC-GC interface. Data confirmed its localization pattern, since, in the control, Cx43 was detected in the above-mentioned cell types, and particularly in SPC (dotted arrows; Fig. 4), SC (arrowheads; Fig. 4; insets) and its cytoplasmic protrusions surrounding SPT (arrows; Fig. 6E). Moreover, a clear localization in the LC (asterisks; Fig. 4) was appreciable. Cd treatment provoked a marked decrease of staining in GC, while a weak signal was still present in SC cytoplasm (arrowheads; Fig. 4 and insets) and in the LC (asterisk; Fig. 4). No difference was found between control and Cd+MLT.

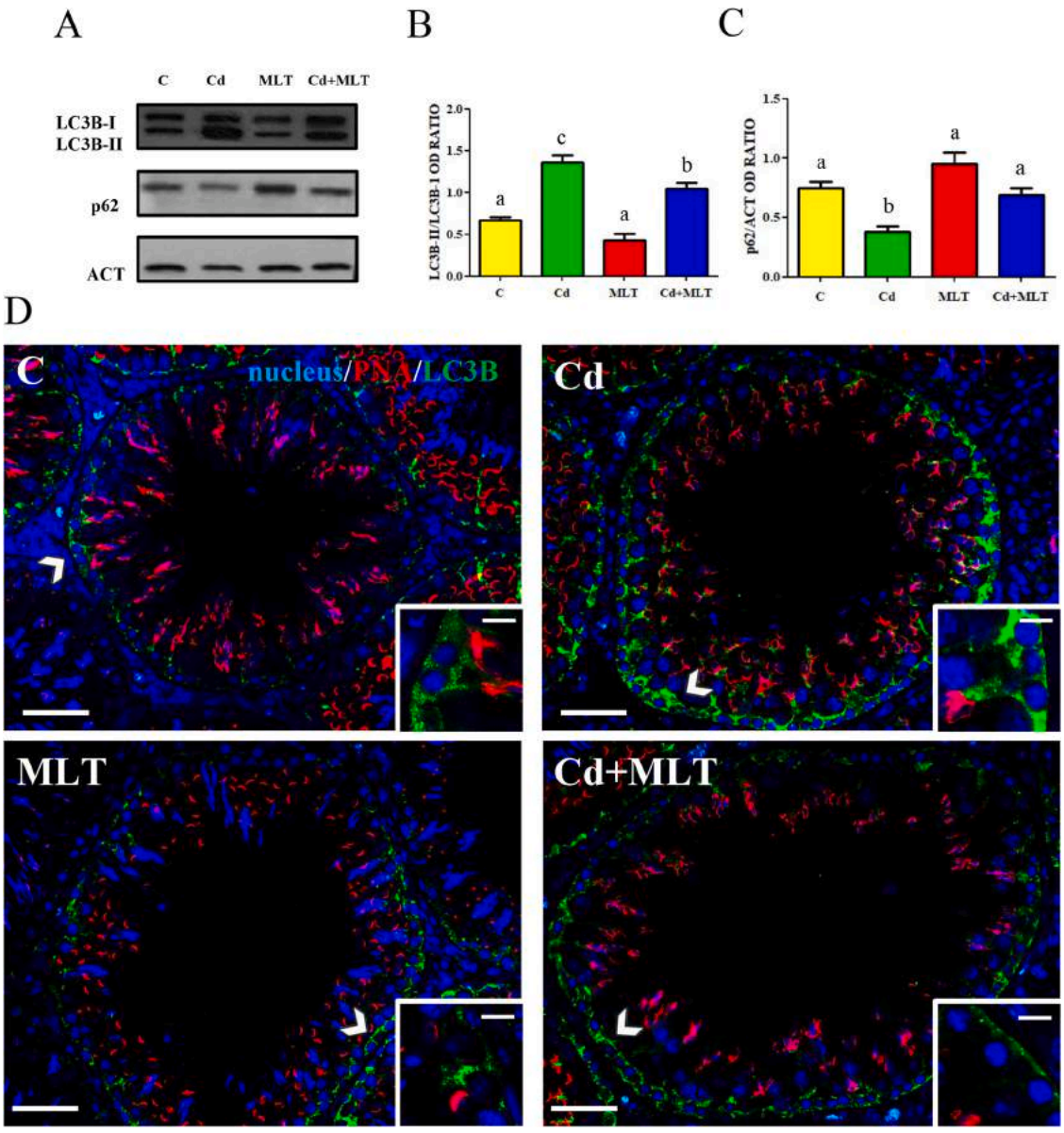

Fig. 1. Autophagy rate analysis of C, Cd and/or MLT treated rat testis. A: WB analysis showing the expression of LC3B-I (14 kDa), LC3B-II (16 kDa), p62 (62 kDa) and β-actin (44 kDa) in testis of animals treated with Cd and/or MLT. B, C: Histograms showing LC3B-II and p62 relative protein levels. Protein levels were normalized with LC3B-I and β-actin, respectively, and reported as OD ratio. All the values are expressed as means ± SEM from 6 animals in each group. Statistical significance was evaluated by ANOVA (at least $p < 0.05$) followed by Tukey test for multigroup comparison. Same letter indicates no significative difference; different letters indicate significative differences. D: IF analysis of LC3B (green) in testis of animals treated with Cd and/or MLT. Slides were counterstained with DAPI-fluorescent nuclear staining (blue) and with PNA lectin (red) which marks the acrosome. Scale bars represent 20 μm, and 10 μm in the insets. Arrowheads: SC. All the WB and IF experiments were performed in triplicate.

3.4. Effect of Cd and/or MLT on sperm physiology

3.4.1. Analysis on sperm parameters, lipid peroxidation and DNA integrity

The effects of Cd and/or MLT treatment on the main sperm parameters are shown in Table 1. Cd produced consistent alterations in all the considered parameters, as compared to the other groups. Particularly, Cd significantly decreased the DSP ($p < 0.05$), sperm concentration ($p < 0.05$), viability ($p < 0.05$), motility ($p < 0.01$) and morphology ($p < 0.001$) as compared to the control. Sperm morphology abnormalities were characterized by detached, double, looped or by absence of head, other than by looped, coiled, curved, reduced, and bent tail (not shown). No difference between Cd+MLT and control was observed in the parameters analyzed. Worthy of note is that MLT alone increased the motility as compared to the control ($p < 0.01$).

The Cd-induced oxidative stress was further assayed analyzing the sperm lipid peroxidation rate through TBARS assay (Fig. 5A). Cd exposure produced a significant increase of TBARS levels when compared to the control ($p < 0.001$). MLT given in combination with Cd completely counteracted the increase in TBARS observed in the Cd group ($p < 0.001$), decreasing it to levels similar to those of control. Finally, MLT alone produced a decrease in the TBARS levels as compared to the control ($p < 0.05$).

Using the acridine-orange staining, the toxic effect of Cd on SPZ DNA integrity was evidenced by a significant increase in the number of SPZ showing a yellow/orange head, as compared to the control ($p < 0.001$; Fig. 5B, C), in which most SPZ, without a damaged DNA, showed a green head. The damage SPZ DNA in the Cd+MLT group was no different from the control.

Finally, the beneficial effects of MLT alone provoked a decrease in SPZ DNA damage, as compared to the control ($p < 0.05$) and Cd+MLT

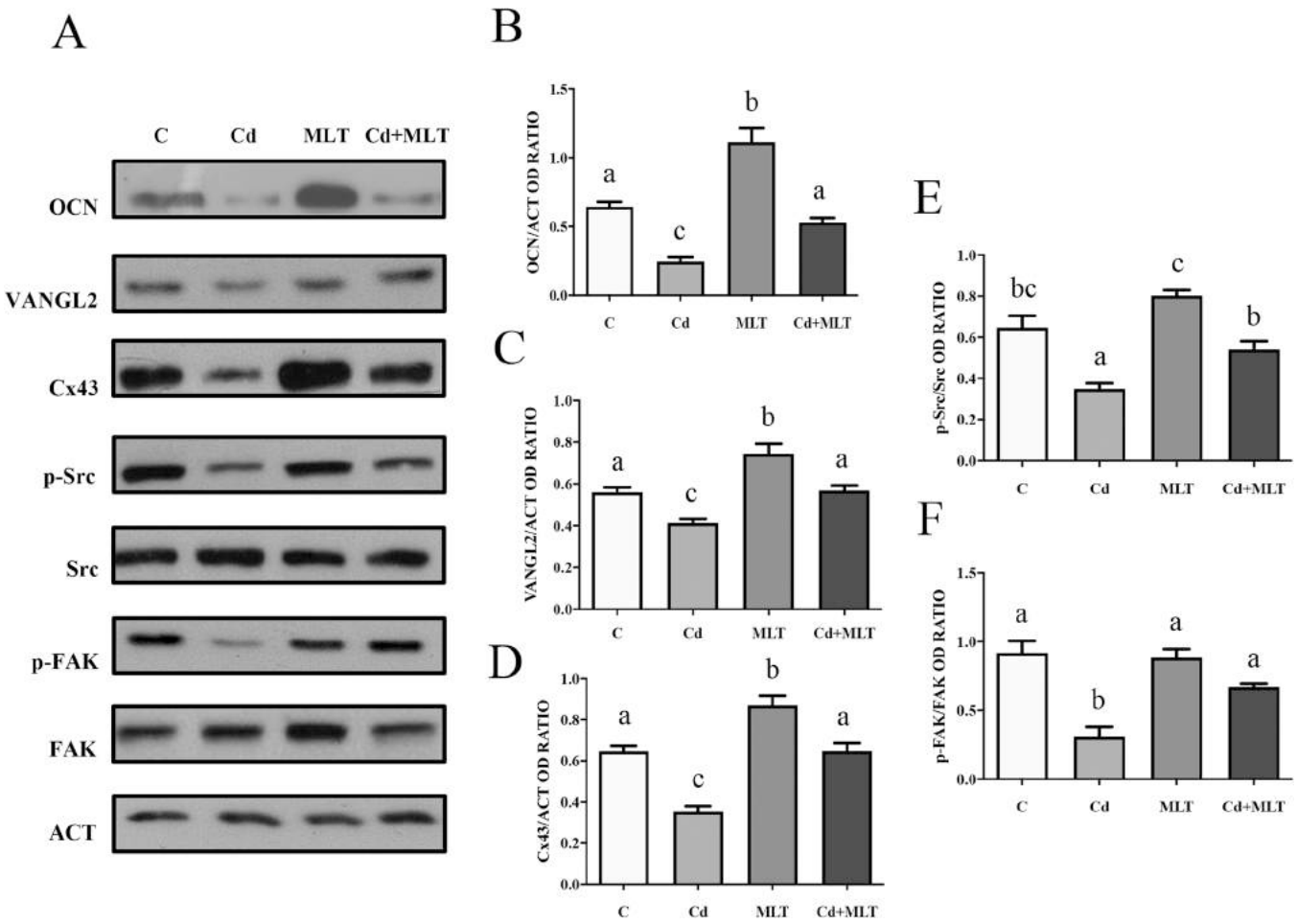

Fig. 2. WB analysis of blood-testis barrier markers in C, Cd and/or MLT treated rat testis. A: WB analysis showing the expression of OCN (59 kDa); VANGL2 (60 kDa); CX43 (43 kDa); p-Src (60 kDa); Src (60 kDa); p-FAK (120 kDa), FAK (120 kDa) and β-actin (44 kDa) in testis of animals treated with Cd and/or MLT. B, C, D, E, F: Histograms showing OCN, VANGL2, Cx43, p-Src and p-FAK relative protein levels. Protein levels were normalized with β-actin or the relative non-phosphorylated form and reported as OD ratio. All the values are expressed as means ± SEM from 6 animals in each group. Statistical significance was evaluated by ANOVA (at least $p < 0.05$) followed by Tukey test for multigroup comparison. Same letter indicates no significative difference; different letters indicate significative differences. All the WB experiments were performed in triplicate.

($p < 0.01$) groups.

3.4.2. Analysis on prolyl endopeptidase (PREP)

To further analyze the effects of Cd and/or MLT treatment on sperm physiology, other parameters where considered. A WB analysis on PREP, a protein involved in the sperm motility, showed that Cd exposure resulted in a significant decrease in its level as compared to the control ($p < 0.001$). No difference between Cd+MLT and control was observed (Fig. 6A, B). Remarkably, MLT alone produced an increased PREP protein level as compared to the control ($p < 0.01$) and Cd+MLT ($p < 0.01$) groups.

To better characterize the effects exerted by Cd and/or MLT on PREP localization, an IF analysis on SPZ was performed (Fig. 6C). PREP clearly localized in the tail, in all the groups, with a lower intensity observed in the sperm of the Cd group.

4. Discussion

From a biological perspective, reproduction is the ultimate purpose of an organism, to preserve and ensure species survival. The production of good-quality gametes is essential for the reproductive success. Currently, human fertility is progressively decreasing since more than 186 million people worldwide are infertile (Bui et al., 2018). Published data highlighted that male contribute to 50% of infertility cases, undoubtedly associated to a declined sperm quality (Bui et al., 2018). Male infertility, above all in the modern world, cannot be traceable only to unhealthy lifestyle, but increasing evidence demonstrated that it is likely due to chronic exposure to environmental pollutants (Gabrielsen and Tanrikut, 2016). Among these, Cd represents a severe threat for two reasons: (1) the continuous exposure of humans to its widespread use in various industrial processes, and Cd contamination of food/water and tobacco cigarettes (Wang et al., 2021a); (2) Cd has an extremely long half-life in the human body (20–40 years); thus, even if the exposure occurs at low dose, it can bio-accumulate in many tissues (Wang et al., 2021a).

Previous studies demonstrated that rat testis is a suitable model to investigate not only the Cd-induced toxicity in spermatogenesis, but also to verify new strategies to counteract its action (Unsal et al., 2020) to obtain useful information that may be applied to humans. The MLT ability to offer a defense against Cd actions has been demonstrated in different rat tissues, i.e. bones (Knani et al., 2020); brain (Lamtai et al., 2021), ovary (Kechiche et al., 2021) and testis itself (Venditti et al., 2021a). Herein, we evaluated the possible effect(s) of MLT against the Cd effects on the blood-testis barrier and on sperm quality.

It is well known that Cd adverse influence on testicular function is probably due to the induced oxidative stress, as supported by the increased TBARS levels in SPZ, an index of the lipid peroxidation (Lama et al., 2019). Per se, Cd is unable to generate free radicals and the

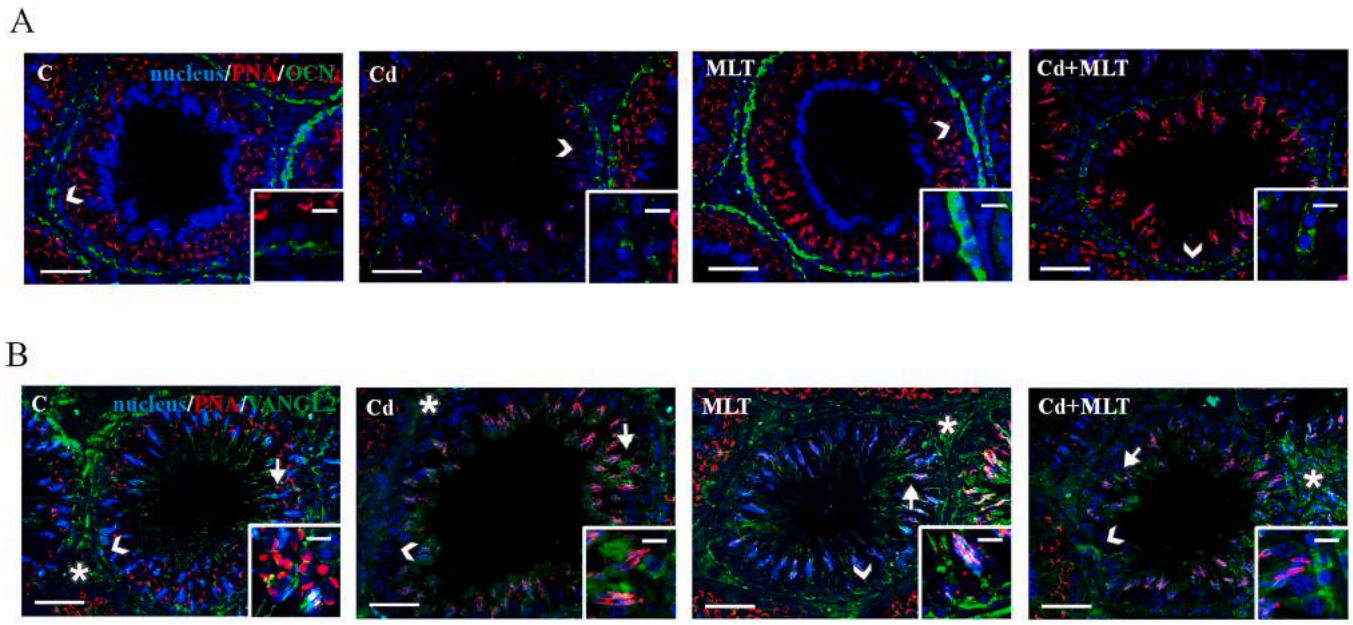

Fig. 3. IF analysis of OCN and VANGL2 in C, Cd and/or MLT treated rat testis. A: IF analysis of OCN (green). Slides were counterstained with DAPI-fluorescent nuclear staining (blue) and with PNA lectin (red) which marks the acrosome. B: IF analysis of VANGL2 (green). Slides were counterstained with DAPI-fluorescent nuclear staining (blue) and with PNA lectin (red). Scale bars represent 20 μm, and 10 μm in the insets. Arrows: SPT; Arrowheads: SC; Asterisk: LC. All the IF experiments were performed in triplicate.

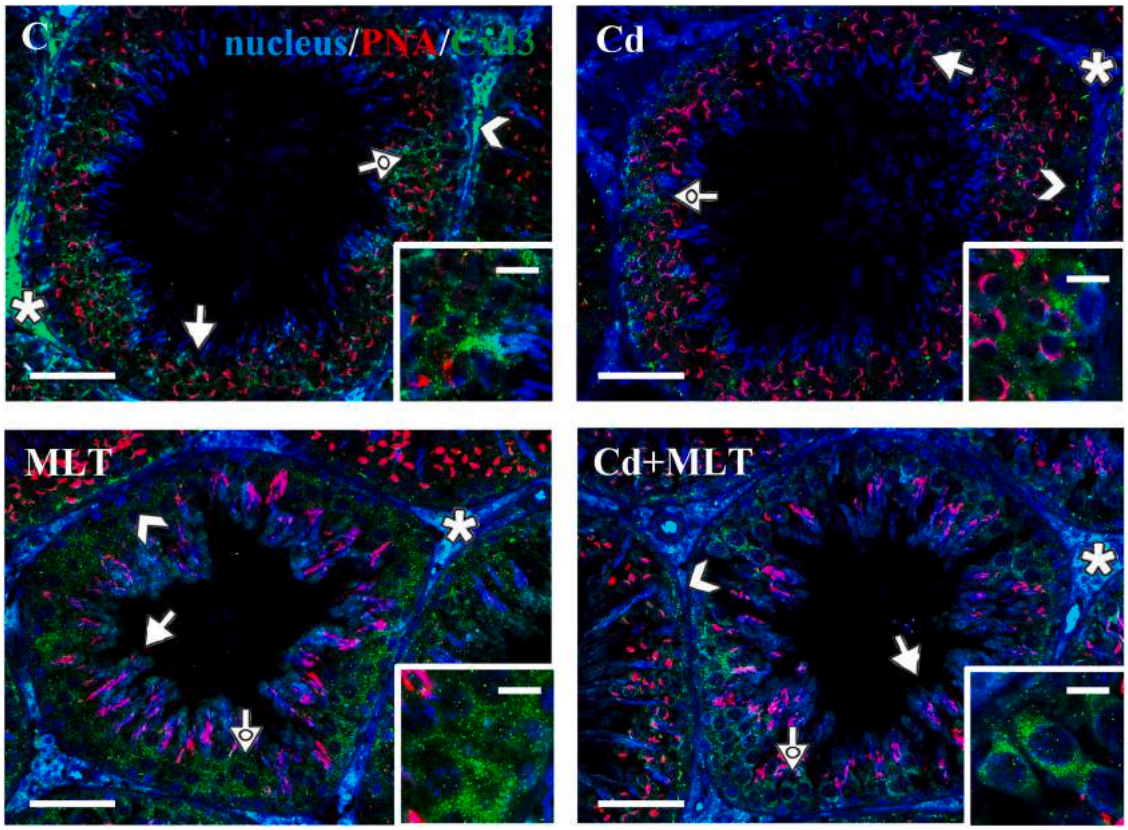

Fig. 4. IF analysis of Cx43 in C, Cd and/or MLT treated rat testis. IF analysis of Cx43 (green). Slides were counterstained with DAPI-fluorescent nuclear staining (blue) and with PNA lectin (red). Scale bars represent 20 μm, and 10 μm in the insets. Dotted Arrows: SPC; Arrows: SPT; Arrowheads: SC; Asterisk: LC. All the IF experiments were performed in triplicate.

induced oxidative stress may occur through two mechanisms: (1) via the molecular mimicry phenomenon, Cd replaces other metals, such as iron and copper, in cytoplasmic and membrane proteins, thus increasing their concentrations; this, in turn, induces ROS overproduction by the Fenton reaction; (2) Cd interferes with the –SH groups within the active sites of the antioxidant enzymes affecting their activity. Considering that

Table 1
Effect Cd and / or MLT on sperm parameters.

Groups	C	Cd	MLT	Cd-MLT
DSP/g testis (10^6)	15.81 ± 0.42	11.46 ± 1.16[a]	17.61 ± 0.3[b**]	14.8 ± 1.45[b]
Sperm concentration (10^{-6}/mL)	11.13 ± 0.67	8.46 ± 0.62[a]	12.13 ± 0.77[b**]	11.25 ± 0.83[b]
Viability (%)	93.97 ± 2.03	88.1 ± 1.21[a]	97,75 ± 0,45[b**]	96,59 ± 1,05[b*]
Motility (%)	26.42 ± 0.91	15.85 ± 1.04[a*]	36.57 ± 2.49[a*b**]	28.33 ± 1.78[b**c]
Abnormal morphology (%)	34.62 ± 5.8	65.78 ± 4.15[a**]	28.89 ± 2.77[b**]	32.74 ± 3.35[b**]

Evaluation of sperm parameters of animals exposed to Cd and/or MLT. Values are expressed as mean ± SEM from 6 animals in each group. a: significant difference versus C group, $p < 0.05$; b: significant difference versus Cd group, $p < 0.05$; c: significant difference versus MLT group, $p < 0.05$; *: $p < 0.01$; **: $p < 0.001$.

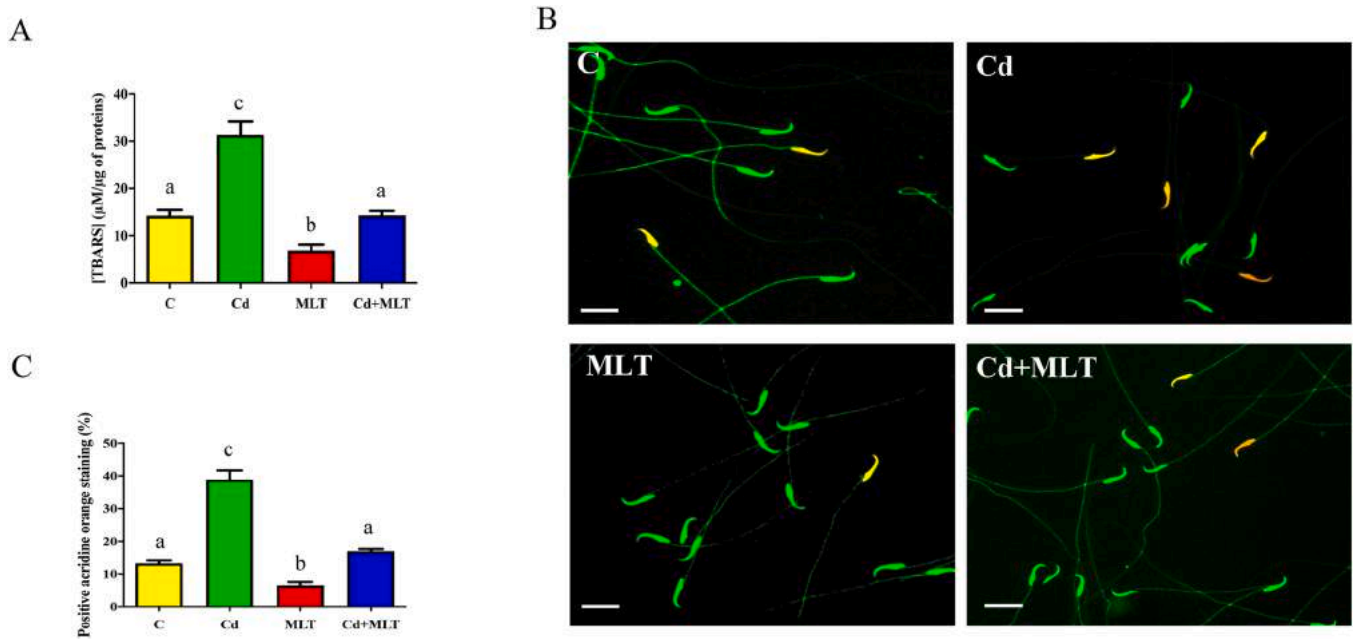

Fig. 5. Sperm physiology analysis of C, Cd and/or MLT treated rat testis. A: Histogram showing TBARS levels of animals exposed to Cd and/or MLT. B: Acridine orange staining, that highlight the SPZ with damaged DNA (yellow/orange) respect to those with intact DNA (green). C: Histogram showing the percentage of SPZ with damaged DNA. In B and C, all the values are expressed as means ± SEM from 6 animals in each group. Statistical significance was evaluated by ANOVA (at least $p < 0.05$) followed by Tukey test for multigroup comparison. Same letter indicates no significative difference; different letters indicate significative differences. All the experiments were performed in triplicate.

testicular cells and SPZ have a high content of unsaturated fatty acids, testes are extremely susceptible to ROS (Dutta et al., 2019).

Moreover, the evidenced reduction in serum/testicular T concentrations, confirmed the endocrine disrupting action of Cd (de Angelis et al., 2017). Additionally, T regulates spermatogenesis, acting as a "survival factor" to protect GC from apoptosis, its depletion provoked the reduced relative testicular weight, the altered histological structure and of the sperm parameters (Venditti et al., 2021a, 2021b).

T regulatory actions on spermatogenesis is not limited to GC proliferation/differentiation (Venditti et al., 2020a), but also to several unique events occurring in the BTB (Mruk and Cheng, 2015). It is well known that, during the SE cycle BTB undergoes to a concurrent restructuring at SC-SC (basal BTB) and SC-SPT (apical ES) interface, allowing the transit of GC towards the SE and SPZ release into the lumen (Cheng and Mruk, 2010). T stimulates the synthesis of structural proteins, including OCN (Cheng and Mruk, 2010) as well as the phosphorylation status of some regulatory kinases, i.e. Src (Xiao et al., 2019) and FAK (Gungor-Ordueri et al., 2014). Thus, all the BTB components work harmoniously through continuous cycles of phosphorylation/de-phosphorylation, endocytosis of membrane proteins and their re-cycling, ensuring the correct transport of GC and the maintenance of the immune-privileged microenvironment.

Increasing evidence demonstrates that Cd perturbs the BTB physiology (Ramos-Treviño et al., 2017). Herein, we confirm that Cd alters Src and FAK phosphorylation (Wong and Cheng, 2011), as well as interfering with the level of OCN protein, a tight junction integral membrane protein that interacts with homologous/heterologous OCN localized on adjacent SC (Chung and Cheng, 2001). Cd affects the phosphorylation status of OCN/ZO-1 complex FAK-regulated, inducing their redistribution supported by endocytosis of OCN (Su et al., 2011).

To our knowledge, this is the first study reporting that Cd affects testicular protein level and localization of Cx43 and VANGL2, proteins forming the gap-junctions and the ES, respectively. Among the testicular gap-junctions, Cx43 is the most prominent representative (Li et al., 2010), and it provides the necessary coordination of cellular events to ensure the synchronization of GC development throughout the tubule (Cheng et al., 2011); furthermore, its presence in LC may indicate a role in paracrine communication between germinal and interstitial compartments (Palmiero et al., 2003). Finally, Li et al. (2010) demonstrated that Cx43 is critical for the maintenance and reassembly of the transiently disrupted BTB during the epithelial cycle.

Regarding VANGL2, this Planar Cell Polarity protein plays a central role in regulating BTB integrity and SPT transport, acting on actin microfilaments and microtubules organization. VANGL2, by controlling the spatio-temporal expression of actin-regulatory proteins and the polymerization of microtubules, ensures the organized alignment of GC, especially SPT, across the plane of SE (Chen et al., 2018a). Worthy of note, an impaired localization at SC-SPT interface was evidenced,

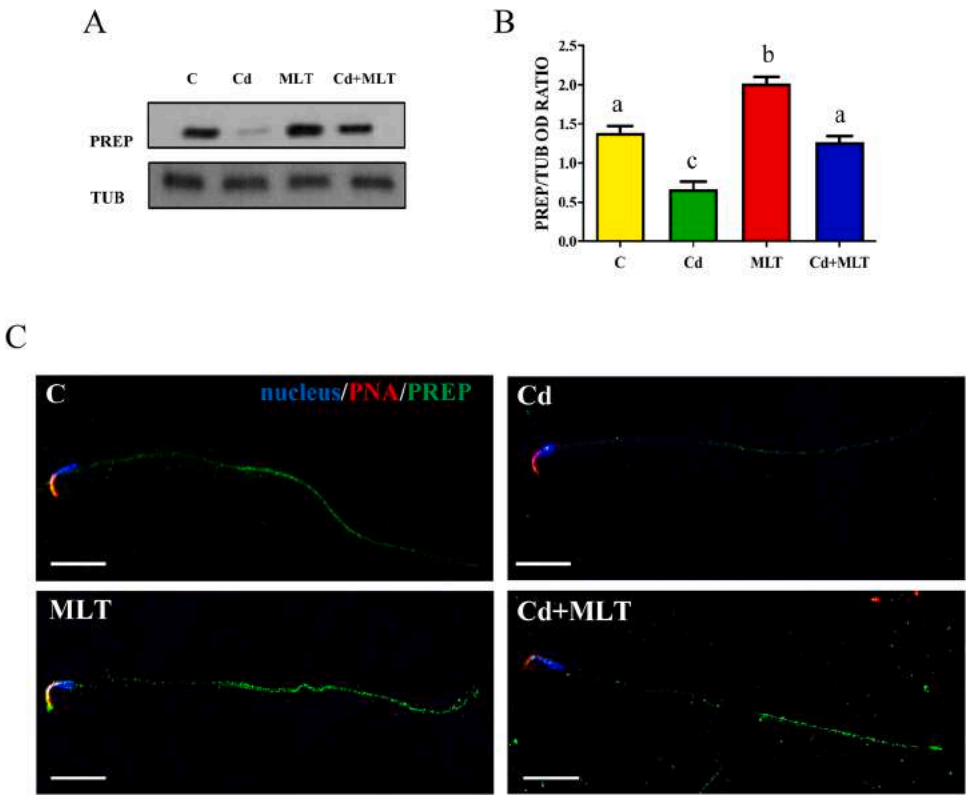

Fig. 6. WB and IF analysis of PREP in C, Cd and/or MLT treated rat SPZ. A: WB analysis showing the expression of PREP (80 kDa) and α-tubulin (50 kDa) in SPZ of animals treated with Cd and/or MLT. B: Histograms showing the relative protein levels of PREP. Data were normalized with α-tubulin and reported as OD ratio. All the values are expressed as means ± SEM from 6 animals in each group. Statistical significance was evaluated by ANOVA (at least $p < 0.05$) followed by Tukey test for multigroup comparison. Same letter indicates no significative difference; different letters indicate significative differences. C: IF analysis of PREP in SPZ of animals treated with Cd and/or MLT. Slides were counterstained with DAPI-fluorescent nuclear staining (blue) and with PNA lectin (red). Scale bars represent 20 μm. All the WB and IF experiments were performed in triplicate.

suggesting that Cd, besides down-regulating VANGL2 expression, may also affect its spatial configuration.

These findings validate that BTB is one of the main targets of testicular Cd toxicity that destabilizes its integrity, leading to the loss of contacts between the cells and their exfoliation. Cd-induced defects at BTB may be due to the withdrawal of T and/or to the activation of the oxidative stress-mediated p38 MAPK pathways (Chen et al., 2018b); however, the involvement of other mechanism(s) is not precluded.

Noticeably, many stressful conditions (oxidative stress or the absence of survival factors) may induce autophagy, an adaptive mechanism in which damaged molecules and organelles are degraded in the lysosomes. Playing prominent roles for the progression of autophagosome formation and maturation are microtubule-associated protein 1A/1B-light chain 3 (LC3) and p62, markers commonly used to monitor the autophagy activity (Yoshii and Mizushima, 2017). Data confirm previous reports demonstrating that Cd induces autophagy (Wang et al., 2020a, 2020b, 2021b; Zhou et al., 2021), with the highest rate in SC. This point is of interest, because it could be linked to the loss of integrity of BTB components, suggesting that, to avoid increasing of BTB destabilization caused either by lack of T or ROS overproduction, proteins composing the junctions are destroyed via autophagy. This mechanism could also explain, at least in part, the reduced protein levels observed in the BTB components, although the influence of Cd on their expression cannot be excluded and future research are needed to clarify this aspect.

Our data strongly suggest that Cd alters male reproductive function and affects the gamete quality; hence, the use of strategies to limit its action is of vital significance to sustain human health. MLT may represent a suitable molecule to counteract Cd toxicity as its antioxidant and antiapoptotic properties are well documented (Reiter et al., 2000). MLT can reduce oxidative stress directly, acting as ROS scavenger, and indirectly, by stimulating the efficiency of several antioxidant enzymes (Reiter et al., 2000), or by activating some pathways, i.e that of Nrf2 (Ahmadi and Ashrafizadeh, 2020) and of SIRT1 (Xu et al., 2020), which are involved in the protection against redox unbalance and DNA damage, respectively. To date, few papers, exclusively focused on oxidative stress and pro-apoptotic effects, highlighted the ameliorative action of MLT against testicular Cd toxicity (Eybl et al., 2006; Ji et al., 2012; Kara et al., 2007; Karbownik et al., 2001; Li et al., 2016). However, recently we demonstrated that MLT was efficient in preserving SOD and CAT antioxidant activity and inhibiting Cd reprotoxicity-induced decrease in expression of two cytoskeleton-associated proteins, DAAM1 and PREP (Venditti et al., 2021a, 2021b). This report presents new information, since it is the first to also document the counteractive effects of MLT on other parameters that have not heretofore been analyzed. Here we demonstrated that MLT nullified Cd influence on all the analyzed parameters, because of restored T serum/testicular concentration, confirming that it preserved testicular function and morphology by maintaining a balanced sex hormone homeostasis. Thus, in this study, all the results obtained further confirmed the ability of MLT in counteracting Cd damages in testicular activity, highlighting that this indolamine, by alleviating the action exerted by Cd, may promote reproductive success, preserving a good sperm quality.

A further result observed is that MLT alone ameliorated several parameters: in the testis, the level of the structural proteins composing the BTB, while in the SPZ the TBARS levels, DNA damage and motility. The effect on the testis is of interest, since previous papers reported that MLT increased the expression of cell junction proteins inhibiting tumor invasion (Zhou et al., 2014); however, more detailed studies are needed to clarify the underlying molecular mechanism(s).

The ameliorative effect on SPZ motility could be the consequence of Ca^{2+} mobilization from intracellular storage sites, dependent by the activation of MT1/MT2 receptors (Pandi-Perumal et al., 2008), together with an increased PREP level, whose activity have been positively correlated to SPZ motility in mouse (Dotolo et al., 2016) and human (Venditti et al., 2020b).

5. Conclusions

This report further documents the protective action of MLT on Cd-induced toxicity on rat testis. This study added new insights into the

mechanism related to the protective role of MLT on all the testicular and sperm quality parameters. This is the first study to show a counteractive effect of MLT on the cytoarchitecture of BTB. In addition, MLT alone preserves sperm quality (DNA damage and motility). The overall results strongly confirm the MLT role in improving rat testicular health, encouraging further studies to verify its action not only in men exposed to Cd, but also in those having fertility disorders, to produce high quality sperm and, consequently, to improve reproductive success.

Ethics approval and consent to participate

The experimental procedure was approved by the Ethics Committee for Research in life science and health of the Higher Institute of Biotechnology of Monastir (CER-SVS/ISBM- protocol 022/2020) and was carried out accordingly to the UNESCO Recommendation Concerning Science and Scientific Research (1974, 2017).

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CRediT authorship contribution statement

Venditti Massimo: Investigation, Formal analysis, Writing – original draft, Supervision, Funding acquisition. **Mariem Ben Rhouma**: Methodology, Formal analysis. **Maria Zelinda Romano**: Investigation. **Imed Messaoudi**: Conceptualization, Methodology, Validation. **Russel J. Reiter**: Validation, Visualization, Writing – review & editing. **Sergio Minucci**: Conceptualization, Funding acquisition, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Code availability

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112878.

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