Metallothionein expression and synthesis in the testis of the lizard *Podarcis sicula* under natural conditions and following estrogenic exposure

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Abstract

Metallothionein (MT) is the main protein involved in the homeostasis of metallic micronutrients and in cellular defence against heavy metals and reactive oxygen species. Found in almost all vertebrate tissues, MT presence and localization in the testis has been controversial. In the present study, by using *in situ* hybridization and immunohistochemical analysis we assessed the localization of both MT transcript and protein in *Podarcis sicula* testes during two different phases of the reproductive cycle: the autumnal resumption and the spring-early summer mating period. In addition, with the same methodological approach, we verified the effect of estradiol-17β and nonylphenol, a potent xenoestrogen, on MT expression and synthesis. These results, the first collected in a non-mammalian oviparous vertebrate, demonstrated that the expression profile of MT mRNA and protein changes during the reproductive cycle. In the fall resumption, MT transcripts are absent in spermatogonia and present in all the other cells of tubules, including spermatozoa; vice versa, the MT protein is evident only in spermatozoa and somatic cells. In the mating period, both MT transcripts and proteins are present in spermatogonia, spermatocytes and spermatids, whereas in the spermatozoa only the proteins are detected, thus suggesting that the MTs translated in the earlier germinal stages are stored up to spermatozoa. Results also demonstrated that in lizard testis the expression of MT gene undergoes a cell-specific regulation after estrogenic exposure; the possible role and the mechanism by which this regulation occurs have been discussed.

Introduction

Metallothioneins (MTs) are low molecular weight (6-7 kDa), cysteine-rich (~30%) proteins belonging to a multigene family widely distributed in animals, plants, fungi and some prokaryotes.[1] These molecules play a key role in the homeostasis of essential metallic micronutrients such as zinc and copper; they are also involved in cellular adaption to stress, being considered scavenger of reactive oxygen species (ROS), and in detoxification of nonessential toxic metals.[2] Their metal-detoxifying effect is due to the ability to bind and store heavy metals in a non-toxic metal-MT complex.[3]

MTs have been physiologically detected in several tissues of both aquatic and terrestrial vertebrates.[1,4-6] Since MTs expression could be induced or affected by heavy metals, irradiations, infections, ROS, alcohols, pharmaceuticals, hormones and hormone-like chemicals[7-10] they are considered a valid biomarker of cellular insults in many taxa such as invertebrates, fish and tetrapods, including mammals.[6,7,11,12] MT transcription and synthesis has been also observed in invertebrate and vertebrate embryos.[13-16]

Interestingly enough, there is a long-standing controversy on the presence of MT in the testis. In the past years, many authors sustained the lack of MTs in the male gonad.[17] At the same time, some studies demonstrated the presence of MT mRNA and/or MT protein in the testis;[18-22] however, contradictory results have been found on the cellular localization of MT-mRNA and protein in testis. Indeed, Danielson and co-workers[18] evidenced MT proteins only in Sertoli and interstitial cells of rat testis, whereas Nishimura and co-workers,[19] in the same species, reported the absence of MT in testicular somatic cells and the presence in spermatogenic cells and mature spermatozoa;[20] Suzuki and co-workers[21] showed the presence of MT protein in the head and in the proximal portion of the tail of rat spermatozoa. High levels of MT transcripts were found in both mouse and rat testes often without a colocalization between mRNA and protein.[23] In rat testis, MT-mRNA detected by digoxigenin-labeled probe was observed only in primary spermatocytes, secondary spermatocytes and spermatids, whereas the protein was detected also in spermatozoa.[20] In mouse, the level of MT mRNA was high in the spermatids and low in the mature sperm cells.[24]

The presence of MT mRNA and proteins in the testis has been also detected in animals experimentally exposed to cadmium, hormones or hormone-like substances, thus suggesting for MTs an important role in spermatogenesis and testes protection against cadmium and xenoestrogens toxicity.[22,25]

Very little information is available on

the MT expression and synthesis under natural, uncontaminated conditions in testis of non-mammalian vertebrates.[22] For this reason, in this study, by using *in situ* hybridization and immunohistochemycal analysis, we decided to investigate the MT expression pattern in seminiferous epithelium of the male lizard *Podarcis sicula* in the mating period (May-June) and during the autumnal resumption (October-November). In this lizard, during the mating period an intense spermatogenic activity occurs and a large number of germ cells in all differentiation stages is present in the seminiferous epithelium; in the summer stasis, seminiferous tubules are composed exclusively of spermatogonia and Sertoli cells only, the autumnal resumption leads to the restarting of spermatogenesis and the appearance in tubules of primary and secondary spermatocytes, spermatids and few non-useful spermatozoa.[26]

It has been also demonstrated that *P. sicula* testis are highly responsive to estrogens or estrogen-like compounds; following estrogenic exposure a general slowdown of spermatogenesis with a failure in the replacement of cells from the basal compartment of the seminiferous tubules is observed, together with a severe impairment of spermatogenesis and alterations in testicular and epididymal structures.[27-29] So,

we have also examined the presence and localization of MT transcripts and proteins in lizard testis after estradiol-17β (E2) or nonylphenol (NP) exposure.

The results showed the presence of both MT transcript and protein in the testis of *Podarcis sicula*; however, a different profile of MT expression and synthesis was observed in the two examined phases of the reproductive cycle. Results also demonstrated that in lizard testis the expression of MT gene undergoes a cell-specific regulation after E2 or NP exposure; the possible role and the mechanism by which this regulation occurs are discussed.

Materials and Methods

Animals and experimental treatment

Adult males of lizard *Podarcis sicula* of field origin (about 7.5-8 cm snout-vent) were caught in the outskirts of Naples (Italy) during the mating period (spring-early summer) and autumnal resumption (October-November), kept in terrariums at natural temperature and photoperiod and fed *ad libitum* with larvae of *Tenebrio molitor*. For the estrogen treatment, animals (n=8) of the mating period received an intraperitoneal injection of E2 (168 ng/100 μL, *i.e.*, 20 ng/g body weight) in reptile physiological solution (NaCl 0.07‰) every second day for 2 weeks. Control animals were injected with the physiological saline solution. For NP treatment, animals (n=8) of the same mating period were fed every other day for two weeks with larvae of *Tenebrio molitor* sprayed with an aqueous NP (Etravon-Syngenta, Italy) solution (0.25%); a drinking trough containing water polluted with NP (0.05%) was always available.[27,30] Control animals were fed with non-polluted food and water for two weeks. At the end of the treatments, all the animals were killed by decapitation after deep anaesthesia with ketamine hydrochloride (Parke-Davis, Berlin, Germany) 325 μg/g body weight; testes were quickly removed and immediately processed for the histological and molecular analyses.

The experiments were approved and carried out in compliance with the ethical provisions enforced by the National Committee of the Italian Ministry of Health on in vivo experimentation (Department for Veterinary Public Health, Nutrition and Food Safety, SCN/2D/2000/9213), and organized to minimize animals number and suffering.

Histology

Both testes of each animal were fixed in Bouin's fluid, alcohol-dehydrated, and paraffin-embedded. Sections of 7 μm in thickness were obtained with Reichert-Jung 2030 microtome. Some histological sections were stained with Mallory's trichrome modified by Galgano; other sections were processed by *in situ* hybridization and immunohistochemistry. The results were examined at Nikon-MicroPhot-Fxa microscope.

In situ hybridization

For *in situ* hybridization analysis, the specific *P. sicula* MT cDNA fragment was obtained from *P. sicula* liver mRNA by a RT-PCR strategy, as previously described.[31] In PCR analysis, specific primers designed on consensus motifs of the coding sequences of vertebrate MT were used;[32] the PCR product was purified using the Qiaquick gel-extraction kit (Qiagen, Hilden, Germany) and cloned into the pCR2-TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA). Plasmid containing the *P. sicula* MT coding sequence was used for generation of the DIG-labelled cDNA probe by PCR using the DIG High Prime DNA labeling mix (Roche Diagnostics, Mannheim, Germany).

Sections mounted onto Superfrost Plus slides (BDH) were dewaxed, fixed in paraformaldehyde 4% in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4) pH 7.4 for 20 min and treated with proteinase K (10 μg/mL) at 50°C for 10 min. The probe was used at a concentration of 80 ng/100 μL in hybridization buffer overnight at 50°C in a moist chamber. The slides were washed with formamide 50% and SSC 2x for 30 min, formamide 50% and SSC 1x for 30 min, and formamide 50% and SSC 0.5x for 15 min, washed in 2x SSC for 3 min and in NTP (Tris-HCl 0.1M pH 7.5; NaCl 0.15M), and then incubated in 2% blocking solution (Roche Diagnostics) in maleic acid buffer for 1 h. The sections were kept overnight at 4°C with alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Diagnostics) (1:2500) in blocking solution and rinsed in NTP buffer for 30 min and in NTM buffer (Tris-HCl 100 mM pH 9.5, MgCl 50 mM, NaCl 100 mM) for 30 min. Finally, the sections were kept in the dark at room temperature in the colour detection substrate solution BCIP/NBT (nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, Roche Diagnostics) in NTM until appearance of the colour. The reaction was stopped by rinsing the slides in Tris-HCl 0.1M pH 7.5 and mounted in Acquovitrex (Carlo Erba, Cornaredo, MI, Italy). DIG-labeled MT cDNA probe was generated by PCR using the DIG High Prime DNA labeling (Roche Diagnostics). The negative control sections were obtained by omitting incubation with the probe and processed as previously described.

Immunohistochemistry

The immunoreactive MT localization was carried out by using the Novolink Max Polymer Detection System (Leica Biosystems, Nussloch, Germany), as previously reported.[28] Briefly, after counteracting endogenous peroxidase using Peroxidase Block for 5 min, sections were washed in 50 mM tris-buffered saline (TBS) pH 7.6 and treated with Protein Block for 5 min. Subsequently, the sections were incubated with mouse metallothionein monoclonal antiboby (UC1MT, Thermo Fisher Scientific) (1:400) overnight at 4°C. The reaction was visualized using the Novolink™ Polymer for 30 min and diaminobenzidine (15 μL of DAB Chromogen to 1 mL of Novolink™ DAB Substrate Buffer), according to the manufacturer's procedure. Negative controls of reactions were performed on other slides by omitting the incubation with the primary antibody. The cell nuclei had been counteracted with hemalum.

Results

Localization of MT-mRNA in *P. sicula* testis

In situ hybridization analysis revealed the presence of MT transcripts in both germ and somatic cells (Figure 1); however, a different profile of MT expression was observed in the two phases of the reproductive cycle examined and after estrogenic exposure, *i.e.*, E2 injection or NP polluted food (Table 1). During the autumnal resumption, MT was expressed in primary and secondary spermatocytes, spermatids and spermatozoa, whereas no signal was evident in spermatogonia (Figure 1a). During the mating period, a strong signal was recorded in spermatogonia, primary and secondary spermatocytes and spermatids; no MT transcripts were detected in spermatozoa (Figure 1b). Reaction was always positive in cytoplasm of Sertoli and Leydig cells (Figure 1 a,b).

After the estrogenic exposures, the seminiferous epithelium appeared reduced in thickness and consisted of spermatogonia, primary spermatocytes, very few secondary spermatocytes, elongated spermatids and

spermatozoa (Figure 1 c',d'). In E2-treated samples, MT expression was still detected in primary and secondary spermatocytes, spermatids and spermatozoa; no signal was evident in spermatogonia (Figure 1c). In the animals fed with NP polluted diet, MT-mRNA was localized only in primary and secondary spermatocytes and in spermatids; no MT mRNA was detected in spermatogonia and spermatozoa (Figure 1d). Sertoli and intertubular cells were always positive, regardless of the type of treatment (Figure 1 c,d). No signal was observed on sections incubated by omitting MT cDNA probe (Figure 1ctr).

Localization of MT-protein in *P. sicula* testis

Immunohistochemistry revealed that MT distribution significantly changed in the two different phases of reproductive cycle, as well as following estrogenic exposures (Table 1).

In natural specimens of *Podarcis* collected in the autumnal resumption, among the germ cells the immunoreactive signal was evident only in spermatozoa; no reaction was recorded in spermatogonia, primary and secondary spermatocytes and spermatids (Figure 2a). During the mating period, MT protein was present in all germ cells of the seminiferous epithelium, from spermatogonia to spermatozoa (Figure 2b). In both phases of reproductive cycle Sertoli and Leydig cells were always immunoreactive (Figure 2a,b).

In the animals injected with E2 or exposed to NP polluted diet, the MT distribution did not changes, being present in all the cells of the seminiferous epithelium, despite the evident morphological alterations (Figure 2 c,d).

No immunohistochemical signal was detected on control sections obtained omitting primary antibody (Figure 2 ctr).

Discussion

The aim of this work was to establish the presence and the localization of MT transcript and protein in the testis of *P. sicula* in two representative phases of the annual reproductive cycle and following estrogenic exposure, to highlight the role of this protein during lizard spermatogenesis.

Spermatogenesis is a complex set of events that within the seminiferous epithelium leads the spermatogonia to differentiate in spermatozoa. The normal progression of spermatogenesis requires the integrity and the interaction between the different somatic/germinal testicular components and the hypothalamic-pituitary-gonadal axis. The process is in fact mainly controlled by a balanced crosstalk between gonadotropins, steroid hormones and their receptors; however, other molecules can be involved in the progression of spermatogenesis.[32]

The testis of the lizard *P. sicula* shows a tubular structure like mammals. During the mating period, in late spring, in the seminiferous epithelium all stages of spermatogenesis from spermatogonia to spermatozoa express receptors for androgens and estrogens[26] and a great amount of sperms fills the lumen of the tubules. This period is followed by a summer stasis, characterized by a high E2 level and a block of spermatogenesis, and then by an autumnal resumption, with a low E2 level and a high testosterone level, leading to a renewal of a defective spermatogenesis without spermiation.[26]

Our results demonstrate that the expression profile of MT mRNA and protein changes during the reproductive cycle. In the fall resumption, MT transcripts are absent in spermatogonia and present in all the other cells of tubules, including spermatozoa; *vice versa*, the MT protein is evident only in spermatozoa and somatic cells. This suggests that MTs are transcribed by germ cells during differentiation, but they are translated only in fully differentiated spermatozoa. In the mating period, both MT transcripts and proteins are present in spermatogonia, spermatocytes and spermatids, whereas in the spermatozoa only the proteins are detected, thus suggesting that the MTs translated in the earlier germinal stages are stored up to spermatozoa.

Together, this expression pattern denotes an involvement of MTs in *P. sicula* spermatogenesis under natural conditions. The presence of the protein in all spermatogenetic stages may indicate a key role of MT in germ cells maturation, since this process is dependent on zinc distribution in seminiferous epithelium. It is known that zinc, interacting with spermatozoa, is involved in sperm motility and viability and its deficiency leads to atrophy of germ cells in the seminiferous tubules and to spermatogenesis failure.[33,34] Furthermore, it has been demonstrated a reversible metal exchange between MT and the estrogen receptor zinc finger motifs, thus indicating the MT as an important physiological regulator of intracellular zinc.[35] So, the presence of MT in all spermatogenic cells may ensure the proper zinc availability also required for the correct functionality of estrogen receptors.

Estrogen-induced testicular injury is widely described among vertebrates, including *Podarcis*.[36,37] In male lizards, E2 or estrogen-like environmental pollutant NP causes in testis the slowdown of spermatogenesis, the reduction of the amount of germ cells in the seminiferous epithelium and, surprisingly enough, the biosynthesis of vitellogenin, the main estrogen dependent protein synthesized exclusively in the liver of oviparous females.[37-39] In general, estrogenic substances trigger the rapid production of intracellular ROS determining oxidative damage and subsequent genetic alterations.[40] Spermatozoa, as all the cells living under aerobic conditions, produce ROS mostly generated from physiological

Table 1. Overview on metallothionein localization in *Podarcis sicula* testis.

| Type of cell | Autumnal resumption | | Spring/early summer mating period | | | | | |
| | | | Natural | | E2 treatment | | NP treatment | |
	MT mRNA	MT protein	MT mRNA	MT protein	MT mRNA	MT protein	MT mRNA	MT protein
Spermatogonia	-	-	+	+	-	+	-	+
Intermediate (spermatocytes, spermatids)	+	-	+	+	+	+	+	+
Spermatozoa	+	+	-	+	+	+	-	+
Sertoli	+	+	+	+	+	+	+	+
Intertubular	+	+	+	+	+	+	+	+

MT, Metallothionein.

metabolic activity. In humans, however an excessive ROS formation that overcomes the ROS scavenging ability of spermatozoa has been related to male infertility, loss of sperm motility and decreased capacity for sperm-oocyte fusion.[41] In this scenario, the effects of E2 or NP exposure on MT expression and synthesis in the testis of terrestrial vertebrates are poorly investigated.[42] From our data emerges that in the testis of *P. sicula* the MT expression pattern is affected by either E2 or NP treatment; the changes, however, do not concern the presence of the MT-protein in the germinal cells. In the E2-treated males MT-mRNA disappears in spermatogonia, is maintained in spermatocytes and spermatids, and appears in spermatozoa. Therefore, E2 elicits a dual action on MT expression pattern, being able to switch off the MT expression in spermatogonia and to switch on in spermatozoa. The silencing of MT gene in spermatogonia could be the result of an epigenetic regulation on this gene, indirectly aroused by the hormone. It is known that a large amount of ROS induced by the E2 treatment might cause the MT gene hypermethylation. Site-specific DNA hypermethylation by ROS is well described,[43] as well as the MT gene hypermethylation as a mechanism of gene regulation during embryo development.[44] However, it cannot be excluded that the MT-mRNAs detected in spermatocytes and spermatids are retained from the spermatogonia by which these differentiating cells arisen. On the other hand, the induction of the MT gene in spermatozoa, demonstrated by the presence of newly transcribed mRNA, may represent a cellular response to higher amount of ROS. The increased biosynthesis of MTs, acting as ROS scavenger, might preserve the fertility of ejaculating spermatozoa. Unfortunately, at the moment it is not known whether the spermatozoa present in testis of E2-treated animals are able to fertilize. Interestingly, MT transcripts are present also in not useful spermatozoa observed during the autumnal resumption stage.

In the animals exposed to NP polluted diet, the MT-mRNA is absent in lizard spermatogonia, as observed for the E2-treated males, but the MT gene is still silent in spermatozoa, as in control animals. It can be postulated that these spermatozoa are not damaged by the lower estrogenic action of NP, so they do not need to switch on the MT gene; however, it cannot be excluded that lizard spermatozoa failed to trigger the MT defence system in presence of NP, thus being damaged. Indeed, it has been demonstrated that NP is able to cross the blood-testis barrier[45] and harms spermatozoa func-
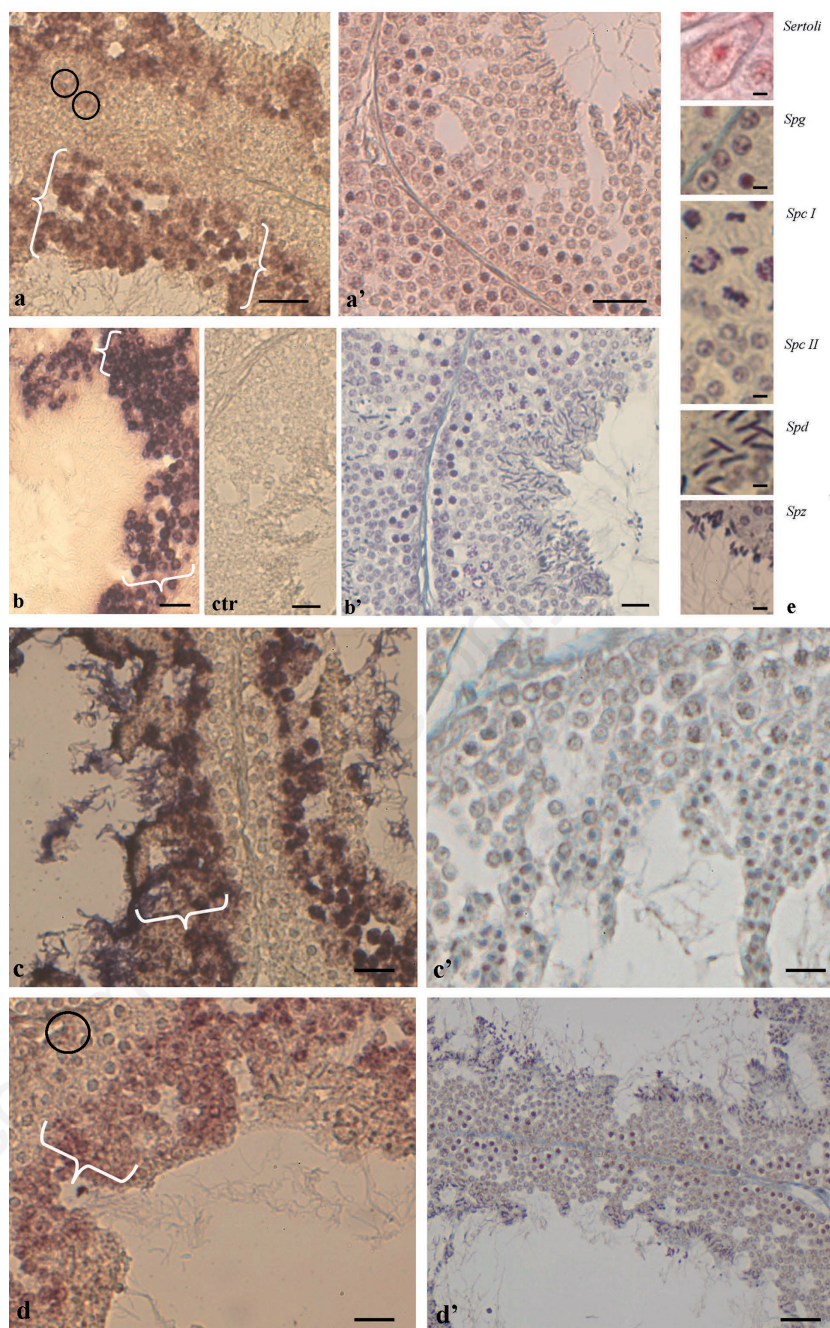

Figure 1. a,b,c,d) MT mRNA localization in testis of natural and estrogen exposed *P. sicula.* **a',b',c',d')** Testis section stained with Mallory's trichrome. **a,a')** Autumnal resumption: **(a)** MT transcripts are present in intermediate cells (*i.e.,* spermatocytes I, spermatocytes II and spermatids, curly bracket), spermatozoa and Sertoli cells (○), no mRNA is evident in spermatogonia present at the basis of the tubules; **(a')** all stages of spermatogenesis are present in the seminiferous epithelium, only few spermatozoa are in the lumen. **b,b')** Mating period: **(b)** MT-mRNA is expressed from spermatogonia to intermediate cells (curly bracket), no reaction is evident in spermatozoa; **(b')** testis section showing the presence of the germ cells from spermatogonia to spermatozoa abundant in the lumen. **c,c')** E2-treated testis: **(c)** MT transcript is evident in intermediate cells (curly bracket), whereas no reaction is evident in spermatogonia at the basis of the tubules; **(c')** seminiferous epithelium with several empty spaces among the germ cells. **d,d')** NP-treated testis: **(d)** MT-mRNA is localized in Sertoli cells (○) and, among germ cells, only in intermediate cells (curly bracket), no reaction is present in spermatogonia at the basis of the tubules and spermatozoa in the lumen; **(d')** seminiferous epithelium greatly reduced in thickness. **e)** Histological sketch representing an overview of the seminiferous tubules in Podarcis; cell types from wall to lumen: Sertoli; spg, spermatogonia; spc I, primary spermatocytes, spc II, secondary spermatocytes; spd, spermatids; spz, spermatozoa. No reaction is evident in the control of reaction (ctr). Scale bars: 30 µm.
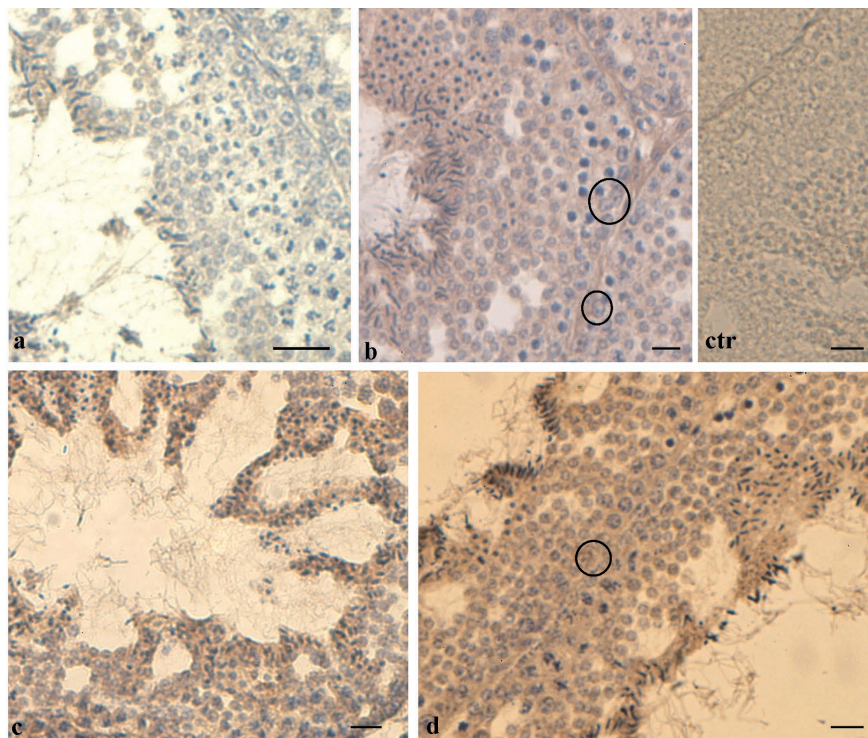
Figure 2. MT protein localization in testis of natural and estrogen exposed _P. sicula_. During the autumnal resumption (a) the immunohistochemical signal (brown areas) is detected only in spermatozoa. In the mating period (b), in E2- (c) or NP- (d) treated males, the signal is evident in all the germ cells present in the seminiferous tubule. See figure 1e for details on the histology of _P. sicula_ seminiferous epithlium. Also Sertoli (○) and intertubular cells are positive to MT antibody. No signal is evident in the control section (ctr). The cell nuclei are counterstained with hemalum. Scale bars: 30 μm.

tionality leading to the loss of their fertilizing capacity in rat, boar and ram.[46]

Finally, results show the presence of MT transcript and protein in somatic cells forming testis, _i.e.,_ Sertoli and intertubular cells, in both the two phases of spermatogenetic cycle and in E2- and NP-treated lizards. These data are in contrast to those reported in mammals, where no MT-mRNA was detected in testicular somatic cells and MT-protein was evident only in Sertoli cells.[20]

Taken together, these results for the first time gather information on the expression and synthesis of MT in the male gonad of a terrestrial oviparous vertebrate. The presence of MT in the testis during spermiogenesis and spermiohistogenesis can be read as a cellular resource ensuring also the accurate interplay between ERs and Zn, thus guarantying correct germ cells maturation. In addition, results also demonstrate that in lizard testis the expression of MT gene undergoes a cell-specific regulation in the animals treated with estrogen or estrogen-like substances; if this regulation is determined, directly or indirectly, by estrogens is yet to be proven.

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