A Preliminary Study of Cadmium Effects on the Adrenal Gland of the Lizard *Podarcis sicula*

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Abstract: Cadmium (Cd) is a heavy metal that can act as endocrine disruptor. Cadmium has the property to accumulate in several organs after entering the body and it principally accumulates in the adrenal glands. Although the uptake mechanisms for the cellular accumulation of Cd are unknown, the most common hypothesis states that Cd uptake involves competition with essential elements such as Ca or Zn for specific transport systems. Cd induces several effects such as cell death, carcinogenesis and disruption of neurotransmitter and hormone action. Particularly, cadmium is able to alter adrenocortical function inducing an impaired capacity to secrete cortisol by steroidogenic cells of the adrenocortical tissues and stimulating catecholamine secretion. In the present paper, we investigated the effects of cadmium exposure on the adrenal gland morphology of the lizard *Podarcis sicula*. For this purpose, we performed two different treatments in order to investigate cadmium effects after both acute and chronic treatments. We have demonstrated that cadmium has toxic effects on the lizard *Podarcis sicula*. Specifically, cadmium induces, in a time-dependent manner, steroidogenic cord hyperplasia, disorganization of steroidogenic parenchyma until necrotic degeneration that in turn evokes macrophage infiltration.

Keywords: Cadmium, endocrine disruptors, adrenal gland, bioindicators.

INTRODUCTION

Cadmium (Cd) is an ubiquitous metal pollutant that can alter the aquatic and terrestrial environment [1]. Its many industrial uses result in large dispersion throughout ecosystems [2]. Cd enters the food chain and, because of its long half-life, is concentrated in various organisms from teleost fish to humans [1,3,4]. Exposure to Cd occurs through intake of contaminated food or water, or by inhalation of tobacco smoke or polluted air [3,5,6]. Moreover, it has been demonstrated that in the leatherback turtle (*Dermochelys coriacea*) the migration in the Atlantic Ocean could facilitate exposure to environmental toxicants like Cd [7]. Particularly, in reptiles, ovo-exposure to Cd has been shown to affect hatchling growth, foraging efficiency, mortality, thyroid function or later reproduction [7-10]. So, in reptiles Cd is particularly toxic in the growth and the tissue development of embryo [7,11]. The tissues that accumulate Cd include the kidneys [12], lung [3,5], reproductive organs [13] and nervous system [14,15]. Moreover, it has been demonstrated that cadmium can accumulate in the adrenal gland after entering the body [16]. The order of important organs that contain cadmium is adrenal gland > liver > kidney > hypothalamus > cerebral cortex [16,17]. However, uptake mechanisms responsible for the cellular accumulation of Cd remain to be identified. The most common hypothesis states that Cd uptake involves competition with essential elements such as Ca or Zn for specific transport systems [1]. Cd causes genotoxicity, teratogeny and cancer in some tissues and nonmalignant chronic toxicity in others [6,7,12,18]. For example, in the lungs Cd is linked to cancer [5], in the kidneys long term accumulation of Cd causes mainly nephrotoxicity [7,12] and in the nervous and endocrine systems Cd disrupts secretion and action of neurotransmitters and hormones, including effects on memory formation [6,13-15,19,20].

The cellular actions of Cd, such as cell death, carcinogenesis and disruption of neurotransmitter and hormone action, are extensively documented, but the molecular mechanisms underlying these actions are still not resolved [3,4,6,21]. It is established that Cd has pleiotropic effects including the induction of immediate early genes (c-fos, c-jun) and genes encoding for metallothioneins and heat shock proteins [4], induction of oxidative stress [4,18] and interference with calcium signaling [6,22,23].

Cadmium, such as some other environmental pollutants, can act as endocrine disruptors [1]. Moreover, recent studies provided evidence that the hypothalamo-pituitary-adrenocortical (HPA) axis, crucial for the ability of vertebrates to cope with stressors, is one of the targets of both metals and endocrine disruptors in several animal species [24]. Particularly, several studies have shown that

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fish sampled at contaminated sites have an altered adrenocortical function with an impaired capacity to secrete cortisol by steroidogenic cells of the adrenocortical tissues [1,25-27]. Furthermore, it has been demonstrated that Cd may act directly on isolated steroidogenic cells in rainbow trout, *Oncorhyncus mykiss* and yellow perch, *Perca flavescens* [1,24]. Several studies demonstrated that Cd, as well as zinc, alter signal transduction pathways leading to cortisol secretion most probably at steps downstream from cAMP formation following stimulation by the adrenocorticotropic hormone (ACTH) [1,24,28]. In mammals, it has been shown that ACTH action is not exclusively mediated by cAMP but also by Ca^{2+} which plays a key role by close interactions through positive feedback loops enhancing steroid secretion [1,29]. Moreover, adrenal gland are responsible for the synthesis and secretion of both adrenaline and noradrenaline into the blood by chromaffin cells. It has been demonstrated that Cd increases intracellular Ca^{2+} *via* generation of 1,4,5-IP3 in cultured bovine adrenal chromaffin cells [30]. In addition, in cat chromaffin cells Cd at micromolar concentration stimulates catecholamine secretion *via* Ca^{2+} [6,19,31].

Bioindicators are critical for assessing both the status and trends in contamination over a wide geographical and temporal scales [32-36]. A good indicator should be easy for scientist to measure, for managers to use and for regulators to employ in compliance mandates [36]. Reptiles could serve as useful bioindicators because many species are widespread, relatively long-lived, occur in a variety of habitats and inhabit a range of trophic levels [8,36-41]. Although most studies of bioaccumulation and trophic transfer deal with aquatic ecosystems, and with organisms that live entirely in the water environment [36], lizards that use terrestrial resources are particularly useful indicators of terrestrial contamination.

Drawing from this background, in the present paper we investigated the morphological effects of cadmium administration on adrenal gland of the lizard *Podarcis sicula*.

MATERIALS AND METHODOLOGY

Animals and Housing Conditions

All the experiments have been carried out in compliance with the ethical provisions enforced by the European Union and authorized by the National Committee of the Italian Ministry of Health on *in vivo* experimentation. Adult specimens of *Podarcis sicula* (14–16 g body weight) of both sexes were captured in the neighborhood of Naples during winter season. After capture, lizards were housed in large soil-filled terraria (width 30 x length 60 x height 50 cm), all containing 5 kg of heather, and exposed indoor to natural temperature (8–12 °C) and photoperiod (11 h daylight). Water dishes were present in the terraria, and the animals were fed on live fly larvae daily. Before beginning experimental procedures, captivity lasted twenty days to reverse capture-related stress [42].

Experimental Procedure

The specimens were divided into two batches, the first receiving an acute cadmium treatment and the second receiving the chronic dietary cadmium treatment.

For the acute cadmium treatment, the specimens were divided into four groups, each consisting of 10 animals (5 males and 5 females) as follows:

Group A: animals receiving a single intraperitoneal injection of cadmium solution (2 μg/g body mass) dissolved in reptilian physiological solution (NaCl 0.75%) with an injection volume of 0.1 ml (injections were made between 8.00 and 8.30 a.m.) and sacrificed 2 days after the injection.

Group B: animals treated as group A but sacrificed 7 days after the injection.

Group C: animals treated as group A but sacrificed 14 days after the injection.

Group D: control animals receiving a single intraperitoneal injection of reptilian physiological solution (NaCl 0.75%) and sacrificed 14 days after the injection.

For the chronic dietary treatment, the second batch of animals was fed at alternate days for 60 days with 1 μg CdCl2 per g of body mass: each dose of cadmium consisted in 40 μL of an appropriate solution of CdCl2 delivered directly into the mouth of the animals with a Gilson micropipette. The specimens were divided into four groups, each consisting of 10 animals (5 males and 5 females) as follows:

Group E: animals receiving the above treatment and killed after 10 days.

Group F: animals receiving the above treatment and killed after 30 days.

Group G: animals receiving the above treatment and killed after 60 days.

Group H: control animals receiving 40 μL of tap water at the same time intervals and killed after 60 days.

The animals were fed ad libitum with mealworms and tap water throughout the treatment.

Light Microscopy

The animals were anesthetized and then killed by decapitation, and their adrenals fixed in a mixture of 2.5% potassium dichromate, 1% anhydrous sodium sulphate (buffered at pH 4.1 with 5 M acetate buffer), and 10% formaldehyde as previously described [43]. They were then embedded in paraplast, cut into 7 μm sections, affixed to albuminized slides, and stained with each one of the following solutions: 1) a mixture of eosine aniline blue, buffered at pH 4 with 5 M acetate buffer [44]; 2) Giemsa solution, modified according to Pearse [45]; and 3) Mallory trichromic stain.

Morphometry

Observations were performed using a Zeiss Axioskop microscope; images were captured with a camera attached to an IBM computer running the Kontron Elektronik KS 300 image analysis system. In 20 specimens from each experimental group, processed for L.M., adrenaline cell, steroidogenic cord diameter, and lymphocyte-macrophage cell count were calculated in every tenth longitudinal section from the whole gland of each specimen. All the morphometric evaluations were performed by three

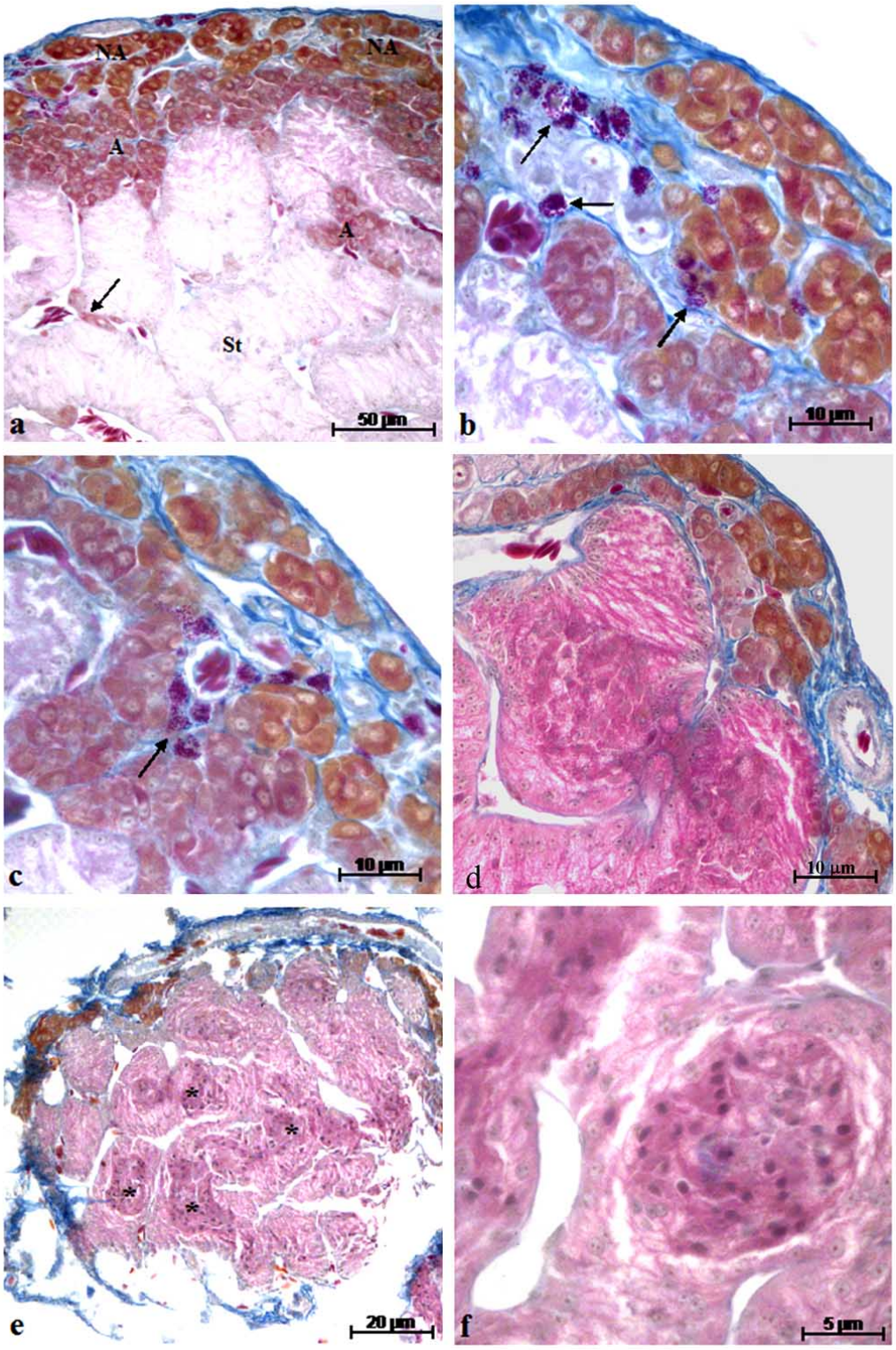

Fig. (1). (**a**) adrenal gland of control specimen. Chromaffin tissue forms a dorsal ribbon, with noradrenaline (NA) cells in the outer layers, and adrenaline (A) cells in the inner layers and islets (arrow) interspersed between steroidogenic (St) tissue, formed by anastomosed cords. Mallory trichromic stain. (**b**) (**c**) adrenal gland of specimen treated with acute cadmium treatment for two days. (**b**) macrophage infiltration (arrows) localized among chromaffin cells of dorsal ribbon. Mallory trichromic stain. (**c**) macrophages assembled close to blood vessels supplying the gland (arrow). Mallory trichromic stain. (**d**) adrenal gland of specimen treated with acute cadmium treatment for seven days. Disorganization of steroidogenic cords inside the gland. Mallory trichromic stain. (**e**) (**f**) adrenal gland of specimen treated with acute cadmium treatment for sixteen days. (**e**) severe steroidogenic disorganization inside the gland with circular clusters (stars) of steroidogenic cell. Mallory trichromic stain. (**f**) high magnification showing the presence of strongly stained nuclei inside the cells forming clusters. Mallory trichromic stain.

Table 1. Incidence of Macrophage Infiltration After Cadmium (Cd) Treatment

Group	Macrophage infiltration
Controls	0/10 (0%)
Acute Cd exposure for 2 days	6/10 (60%) [a]
Acute Cd exposure for 7 days	8/10 (80%) [a]
Acute Cd exposure for 16 days	8/10 (80%) [a]
Chronic Cd exposure for 10 days	8/10 (80%) [a]
Chronic Cd exposure for 30 days	10/10 (100%) [a]
Chronic Cd exposure for 60 days	10/10 (100%) [a]

Number (%) of lizard that presented macrophage infiltration on the total specimens received acute or chronic cadmium (Cd) treatment and control specimens.
[a]Significantly ($p < 0.05$) different from control values.

observers separately. The level of concordance, expressed as the percentage of agreement between the observers, was 96%. In the remaining specimens the opinions of the two investigators in agreement were taken into consideration. The controls and experimental data of all the groups were tested together for significance using Student's t test.

RESULTS

The adrenal gland of the lizard *Podarcis sicula* is formed by an outer dorsal ribbon of chromaffin cells surrounding inner cords of steroidogenic tissue. The steroidogenic cells have a prismatic, weakly stained cytoplasm; the cells are arranged in anastomosed cords of two cell layers, intermingled with blood vessels small in diameter. The chromaffin ribbon contains both noradrenaline (NA) and adrenaline (A) cells; particularly, NA cells occupy the outer layers, whereas A cells are present in the inner layers. Moreover, the chromaffin dorsal ribbon presents many projections, formed by only A cells in the steroidogenic parenchyma. Small groups of A cells, forming scattered islets, are also localized among the steroidogenic cords (Fig. **1a**).

In the specimens receiving the acute cadmium treatment, we observed, after two days, a macrophage infiltration predominantly localized among chromaffin cells of dorsal ribbon and often amid collagen and reticular fibers surrounding the gland (Fig. **1b**). Moreover, macrophages were often assembled close to blood vessels that supply the gland (Fig. **1c**) (Table **1**). After two days, no morphological modifications were observed in the steroidogenic tissue. On the contrary, after seven days, the steroidogenic tissue showed a manifest disorganization of steroidogenic cords (Fig. **1d**). The macrophage infiltration was always present in the chromaffin ribbon. In the specimens treated for sixteen days, we observed a more severe disorganization of steroidogenic parenchyma; particularly, circular clusters of steroidogenic cells were localized at the cord center (Fig. **1e**) (Table **2**). The cells forming these clusters showed a strongly stained nucleus (Fig. **1f**).

In the specimens receiving the chronic cadmium treatment, we observed a weak stimulation of steroidogenic tissue inside the adrenal gland, other than degeneration phenomena similar to acute cadmium treatment. Specifically, after ten days, we observed a faint increase of steroidogenic cord diameter (Fig. **2a**) (Table **2**). Moreover, small clusters of macrophages distributed among chromaffin cells were shown (Fig. **2b**). After thirty days, we observed a weak disorganization of steroidogenic cords, with circular clusters of steroidogenic cells inside them (Fig. **2c**); the constant presence of macrophage infiltration, was shown (Table **1**). After sixty days, the steroidogenic cords appeared more enlarged in diameter with vacuolized cells. Moreover, in several points, the steroidogenic parenchyma showed a severe disorganization; the steroidogenic cells forming circular clusters inside the cords showed strongly heterochromatic nuclei (Fig. **2d**). The macrophage infiltration among chromaffin cells was always present both after thirty and sixty days.

DISCUSSION AND CONCLUSION

Cadmium is a heavy metal dispersed everywhere in the environment, mainly owing to pollution by several sources. The diet is the main source of environmental cadmium exposure in non-smokers in most parts of the world. Atmospheric deposition of airborne cadmium, mining activities and the application of cadmium containing fertilizers and sewage sludge on farm land may lead to the

Table 2. Histomorphometric Analysis of Steroidogenic Tissue After Cadmium Treatment

Group	Steroidogenic cord enlargement	Steroidogenic cord diameter	Steroidogenic cord disorganization
Controls	0/10 (0%)	95 μm	0/10 (0%)
Acute Cd exposure for 2 days	0/10 (0%)	95 μm	0/10 (0%)
Acute Cd exposure for 7 days	0/10 (0%)	95 μm	7/10 (70%) [a]
Acute Cd exposure for 16 days	1/10 (10%)	100 μm	9/10 (90%) [a]
Chronic Cd exposure for 10 days	8/10 (80%) [a]	130 μm [a]	2/10 (20%) [a]
Chronic Cd exposure for 30 days	8/10 (80%) [a]	178 μm [a]	8/10 (80%) [a]
Chronic Cd exposure for 60 days	9/10 (90%) [a]	220 μm [a]	10/10 (100%) [a]

Number (%) of lizard that presented steroidogenic enlargement and disorganization; measure (μm) of steroidogenic cord diameter on the total specimens received acute or chronic cadmium (Cd) treatment and control specimens.
[a]Significantly ($p < 0.05$) different from control values.

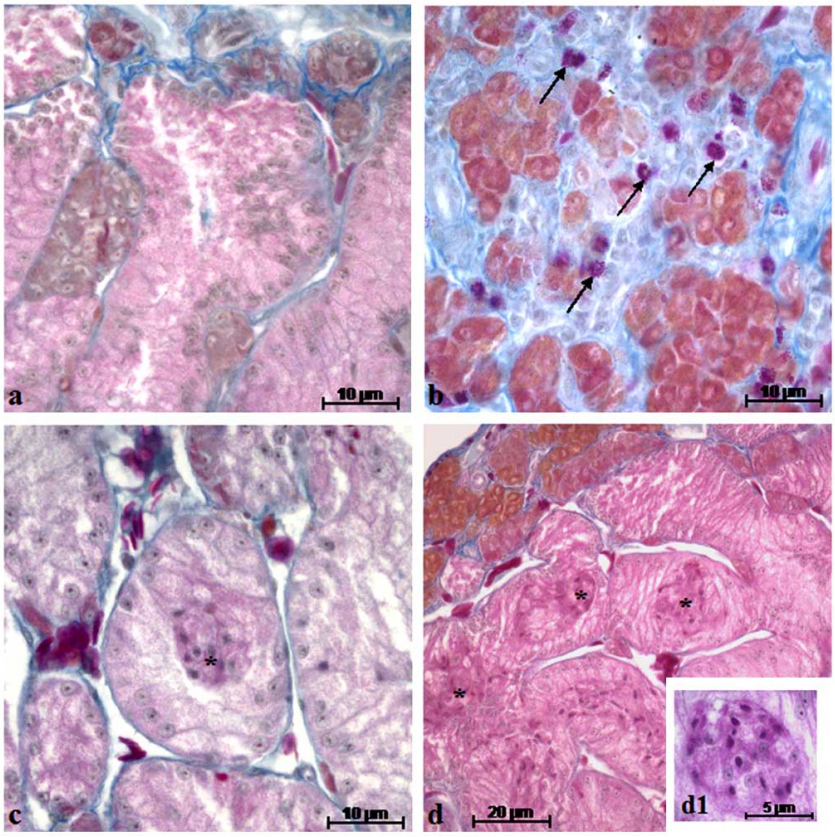

Fig. (2). (**a**) adrenal gland of specimen treated with chronic cadmium treatment for ten days. Light increase of steroidogenic cord diameter. Mallory trichromic stain. (**b**) adrenal gland of specimen treated with chronic cadmium treatment for ten days. Macrophage infiltration (arrows) distributed among chromaffin cells. (**c**) adrenal gland of specimen treated with chronic cadmium treatment for thirty days. Weak disorganization of steroidogenic cords with circular clusters of cells (star). Mallory trichromic stain. (**d**) adrenal gland of specimen treated with chronic cadmium treatment for sixty days. Enlarged steroidogenic cords with vacuolized cells. Note the disorganization of steroidogenic parenchyma (stars). Mallory trichromic stain. (**d1**) high magnification showing the presence of strongly stained nuclei inside the cells forming clusters. Mallory trichromic stain.

contamination of soils and increased cadmium uptake by crops and vegetables grown for human consumption. High concentrations of cadmium are present in molluscs and crustaceans such as oysters and other bivalve molluscs, cephalopods and crabs (especially the parts with brown meat). High levels are also found in offal products such as liver and kidney, especially from older animals, in oil seeds, cocoa beans and in certain wild mushrooms. Food from plants generally contains higher concentrations of cadmium than meat, egg, milk and dairy products and fish muscle. Among food from plants, cereals such as rice and wheat, green leafy vegetables, potato and root vegetables such as carrot and celeriac contain higher concentration than other food from plants [46]. Tobacco smoking is another important source of cadmium exposure. The tobacco leaves accumulate cadmium in a manner similar to certain food from plants. One cigarette may roughly contain 1–2 µg cadmium (varies depending on the type and brand). Roughly 10% of the cadmium content is inhaled with an approximate 50% absorption in the lung. It is estimated that a person smoking 20 cigarettes per day will absorb about 1 µg cadmium daily [46]. The metal has no known beneficial biological function and prolonged exposure to it has been linked to toxic effects in both humans and animals [47]. Cd^{2+} has a long biological half-life of 15–30 years [47], mainly due to its low rate of excretion from the body, and accumulates over time in blood, kidney, and liver [20,48-51]as well as in the reproductive organs, including the placenta, testis, and ovaries [47,51-54]. Specifically, human exposure to the metal is associated with increased incidences of renal disease, hypertension, osteoporosis, and leukemia, as well as cancers of the lung, kidney, urinary bladder, pancreas, breast, and prostate [55].

The vulnerability of the adrenal function of several species to environmental pollutants has been demonstrated by different studies [56-61]. It has been observed that Cd^{2+} interacts with extracellular Ca^{2+} in adrenocortical cells. Particularly, Cd^{2+} competes with Ca^{2+} since the adrenotoxic effect of Cd^{2+} was increased in absence of extracellular Ca^{2+}, but was prevented in media supplemented with Ca^{2+} [61]. In mammalian adrenocortical cells, Ca^{2+} is recognized as a second messenger for ACTH in the signaling cascade leading to corticosteroid synthesis and there is evidence that cytosolic Ca^{2+} concentration is raised by ACTH stimulation [61,62]. It has been demonstrated that the ACTH-stimulated cortisol secretion was abruptly diminished by Cd^{2+}, following the treatment with BAY K8644, an agonist of voltage-dependent calcium channels. Moreover, BAY K8644 pretreatment enhanced Cd^{2+} cytotoxicity estimated by cell mortality [61]. However, the use of a voltage-dependent

calcium antagonist, such as nimopidine, increased adrenotoxicity of Cd^{2+} in adrenocortical cells of rainbow trout suggesting that Cd^{2+} may enter in adrenocortical cells not only trough voltage-dependent calcium [61].

The increased interrenal nuclear size observed for fish living in metal-contamined sites is indicative of chronic stimulation of the hypothalamus-pituitary-interrenal gland (HPI) axis [63]. Several studies have confirmed that histological parameters are good indicators of chronic stress and/or prolonged increase of ACTH secretion [63]. In particular, it has been demonstrated that nuclear diameters increased significantly in stressed sockeye salmon, *Oncorhyncus nerka* [64], as well as in sockeye and rainbow trout, *Oncorhyncus mykiss*, following treatment with ACTH or treatment with the drug metiropone that blocks cortisol synthesis and elevates endogenous ACTH levels [65]. These effects are not induced by short-term treatments [66]. It has been demonstrated that one of the classic responses of fish to chronic stress is interrenal hyperplasia [63-65].

In the present study, we have observed that cadmium, both through the acute and chronic treatment, was able to influence the morpho-physiology of adrenal gland of the lizard *Podarcis sicula*. Particularly, after two days of acute cadmium treatment through intraperitoneal injection of 2 μg/g body weight, we have highlighted the presence of filtering macrophages scattered among both chromaffin cells of dorsal ribbon and among collagen and reticular fibers surrounding the gland. These filtering macrophages probably represent inflammation foci induced by cadmium treatment. After seven and sixteen days of the acute cadmium treatment, we have shown other than an increase of macrophage infiltration, the appearance of a time-dependent disorganization of steroidogenic tissue. This phenomenon was probably due to a necrotic degeneration that in its turn rouses macrophage inflammation. The chronic cadmium treatment, performed by feeding lizards at alternate days with 1 μg CdCl2 per g of body mass, has shown a toxic effect on the lizard adrenal gland. Specifically, we observed after ten days a weak stimulation of steroidogenic tissue in agreement with bibliographic data [63] and the presence of macrophage infiltration. After thirty days, the steroidogenic hyperplasia appears more evident until its peak after sixty days when steroidogenic cells show an increase of vacuoles inside the cytoplasm. This time-dependent effect was evident also for steroidogenic disorganization that appears faint after thirty days until becoming severe after sixty days of treatment. Specifically, the steroidogenic cells form circular clusters inside the cords and show strongly heterochromatic nuclei suggesting the activation of necrotic pathway.

In conclusion, we have demonstrated that cadmium has toxic effects on the lizard *Podarcis sicula* both in the acute and chronic treatment. Specifically, the gland responds to this heavy metal in a time-dependent manner with hyperplasia of steroidogenic cords, disorganization of steroidogenic parenchyma until necrotic degeneration that in turn evokes macrophage infiltration.

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