Cadmium in *Podarcis sicula* Disrupts Prefollicular Oocyte Recruitment by Mimicking FSH Action

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Abstract: Cadmium is a highly polluting heavy metal known to have undesirable effects on health in both animals and humans, targeting the kidneys, the liver and the vascular system. A wide spectrum of deleterious effects has been reported also on the reproductive organs and the developing embryo. Cadmium in fact is a strong endocrine disruptor that interferes with functioning of endogenous receptors and hormones causing detrimental effects on offspring production and survival.

In spite of the wide number of studies carried out in laboratory mammals, data on cadmium effects on gonadic tissues, fertility and reproduction of wild terrestrial vertebrates are still limited. In particular, information on the consequences of environmental cadmium exposure on reptiles survival and biodiversity is particularly scanty. Reptiles are presently considered highly susceptible to a number of environmental pollutants and this has contributed to the global decline of several wild populations of turtles, crocodilians and lizards. In addition, several reptile species have been identified as good bioindicators of pollution in their environments due to their persistence in a variety of habitats, wide geographic distribution, longevity and site fidelity.

In consideration of the few data currently available we decided to investigate cadmium effects on oogonial proliferation and oocyte recruitment in a species of lizard and to verify whether this metal acts as an endocrine disruptor. For this purpose, we treated adult females with cadmium or, alternatively, with estradiol, progesterone or follicle stimulating hormone.

Results indicate that cadmium stimulates oogonial proliferation and oocyte recruitment by mimicking the effects exerted by gonadotropins. Treatment, in fact, increases preleptotene and zygotene-pachytene oocytes numbers that reach values comparable to those observed after FSH treatment. These values are significantly different, either lower or higher, from those observed after estradiol or progesterone administration. Results also indicate that the supernumerary oocytes produced after cadmium treatment are destined to degenerate and that fecundity is reduced by a significant increase in follicular atresia.

Keywords: Lizard, oogonial proliferation, follicular atresia, estradiol and progesterone administration, oocytes hierarchy.

INTRODUCTION

Over recent years, many compounds in the environment have been shown able to mimic or to interfere with the actions of physiological oestrogens [1]. These xenoestrogens are generally acknowledged to be man-made non-steroidal organic chemicals which have been released into the environment from agricultural spraying, industrial processes, urban waste or consumer products and include organo-chlorine pesticides, polychlorinated biphenyls, bisphenol A and phthalates [2-4]. Recently it is emerging that metal ions are also capable of interfering with oestrogen action, so defining a class of inorganic xenoestrogens now termed metalloestrogens [5-7].

The fact that inorganic metal ions also possess oestrogen mimicking properties raises a novel mechanism of endocrine disruption, notable also for the known potential of such materials for wildlife exposure.

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Among metals, various effects of cadmium ions (Cd^{2+}) on reproductive endocrinology have been described [8], but definitive conclusions about cadmium actions on target tissues vary depending on the experimental model and the dosage employed [9]. Exposure of rodents to the metal resulted in a down-regulation of pituitary hormones, including gonadotropins, prolactin, ACTH, growth hormone, and thyroid-stimulating hormone [10]. Similarly, in pseudopregnant rats and in cultured granulosa cells from both rats and humans, Cd^{2+} inhibited progesterone synthesis [11,12].

Impairment of reproductive processes by endocrine disruptors has become a major topic of environmental toxicology in the last few years. In fact, reproduction is a key step in recruitment and stock renewing of species. The most noteworthy reproductive abnormalities related with hormone imbalance are masculinization and feminization in exposed populations [13-15].

In spite of the wide number of studies carried out in laboratory mammals, data on Cd effects on gonadic tissues, fertility and reproduction of wild terrestrial vertebrates are still limited. There is very little experimental laboratory

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research on the effects of Cd in amphibians [16-20], birds [21-23] and reptiles [24-27] and almost no data from studies of wildlife in nature [28]. In particular, it has been demonstrated that Cd exposure decreased survival and metamorphosis in *Bufo americanus* [19] and *Xenopus laevis* [20] tadpoles. Cd can also decrease the thyroid hormone triodothyronine/thyroxine ratios in fence lizards *Scelooporus undulatus* [24], and evidence exists that environmentally relevant doses of Cd may affect gonadal developmental processes of freshwater turtles during embryonic and post-natal stages that may result in disruption of reproductive processes later in life [27].

These data indicate that environmental pollution is one of the main threats affecting the conservation of several reptile and some reptile species have been identified as good bioindicators of pollution in their environments due to their persistence in a variety of habitats, wide geographic distribution, longevity and site fidelity [29,30].

In consideration of the data currently available and in light of the potentially serious consequences of environmental Cd exposure to reptiles survival and biodiversity, we have studied the effect of Cd in the ovary of the lizard *Podarcis sicula* and, in particular, its effects on oocyte recruitment. For comparison animals have been treated with estradiol, progesterone and follicle stimulating hormone. Podarcis ovary is a good model for studying the effects of hormones, drugs and pollutants on oocyte recruitment and selection. In this species, in fact oogonia and prefollicular oocytes are gathered in two small germinal beds [31] and since are rather limited in number (few hundreds) they can be easily counted in serial sections. In addition, this species shows a block of oocyte recruitment into the follicular phases. Every year only a dozen of oocytes become primary follicles and are ovulated. Hence, most oogonia recruited in the oocytes pool undergo atresia between zygotene and early diplotene stages [32].

MATERIALS AND METHODOLOGY

1. Animals

Adult females were captured in the outskirt of Naples, in november, a period in which the ovary is responsive to hormone treatments [33], though in a resting phase. The animals were kept in a terrarium and maintained under conditions of natural temperature and photoperiod, in accordance with the institutional guidelines for care and use of laboratory animals. The lizards were fed live mealworms three times a week and water was provided *ad libitum*. All efforts were made to avoid animal suffering and to minimize the number of specimens used. The experiments were 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 (Dpt. for Veterinary Public Health, Nutrition and Food Safety).

2. Cadmium and Hormone Treatments

For the Cd treatments, animals received a single intraperitoneal injection of a Cd solution, corresponding to a dose of 2 μg/g body weight. For the hormone treatment, a first batch of animals received a single intraperitoneal injection of estradiol (E2, 20μg/g body weight), a second batch received a single intraperitoneal injection of progesterone (P, 25 μg/g body weight) and a third batch a single intraperitoneal injection of follicle stimulating hormone (FSH, 10μg/g body weight). Hormones were from Sigma-Aldrich. Control animals were injected with a physiological saline solution. Sampling was carried out 10 days after the end of treatments.

3. Light Microscopy

Ovaries were dissected, fixed in Bouin's solution and processed for paraffin wax embedding according to routine protocols. Sections were stained with haematoxylin-eosin or Mallory's trichrome to show general morphology. The effects of treatments were verified by counting germ cell in preleptotene, zygotene-pachytene and early diplotene stages, and the number of primary follicles.

4. Atomic Absorption Spectroscopy (AAS)

For the determination of total Cd content, ovaries from control and Cd-treated animals were weighted and digested with concentrated nitric acid (65%, Ultrapure, Fluka), using 1 ml of acid every 50 mg of wet tissue. The mixture was heated for 15 min at 70 °C, cooled and centrifuged for 5 min at 12.000x*g*. Cadmium content in the supernatant was determined by the graphite furnace atomic absorption spectrophotometry, using a Varian atomic spectrometer AA200 equipped with Zeeman graphite furnace. Ultrapure water and stock standard solution of the metal (1 mg/ml) were from commercial source (Fluka). Working standards in 0.2% v/v HNO_3 were prepared daily by diluting known aliquots of the stock solution to the appropriate volume. The detection limit of the metal in different samples was determined from the standard additions curve. It was based on the usual definition of the concentration of the analyte yielding a signal equivalent to three times the standard deviation of the reagent blank signal (n=5). The detection limit estimated was in the range 1-5 ng/g.

RESULTS

Cadmium concentration in the ovaries of control and Cd-treated lizards is given in Fig. (**1**). Data demonstrate that

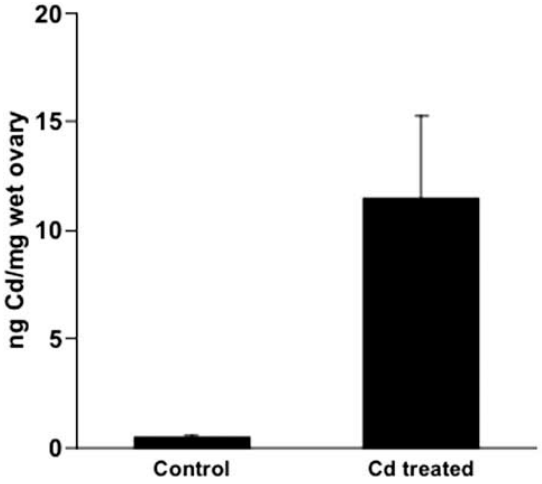

Fig. (1). Cadmium content in ovary of lizards injected with a physiological saline solution (control) or with a single dose of $CdCl_2$ (Cd treated) as described under Methods. Each value is expressed as mean ± S.D. (*n=8*).

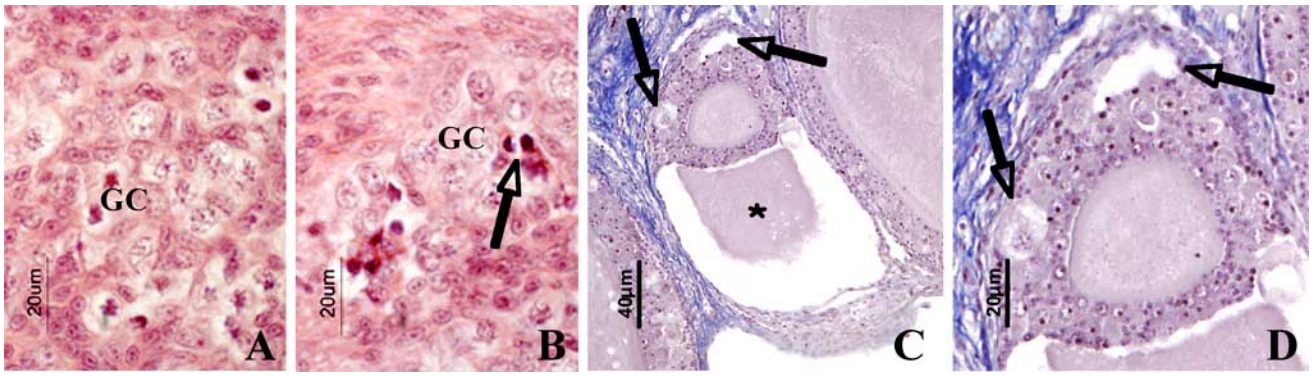

Fig. (2). Effects of cadmium in the ovaries on *Podarcis sicula*. Germinal beds in controls (**A**) and after Cd treatment (**B**); germ cells in differentiation (GC) or in regression (arrow) are clearly recognizable. (**C**-**D**) atretic early previtellogenic follicles in a Cd treated animal. Notice the cytoplasmic remnants (*) of an atretic oocyte and the presence of significant alterations in the epithelium (arrows) of a still intact follicle.

intraperitoneally injected cadmium reaches the ovary, where it accumulates. The presence of a detectable amount of Cd in control ovaries can be easily ascribed to the urbanized sites of capture of wild specimens used for this study.

Cytological observations reveal that in Cd-treated ovaries the germinal beds are particularly large and rich in prefollicular oocytes (Fig. **2B**) as compared to controls (Fig. **2A**). The presence of several pycnotic nuclei suggests that zygotene-diplotene oocytes are undergoing a massive degeneration/selection (Fig. **2B**). Degenerative events are also evident in several previtellogenic follicles (Fig. **2C**,**D**) in which atretic oocytes and/or apoptotic follicle cells can be observed. It is significant that in these stages, in control gonads, atresia is a very rare event: in Podarcis, in fact, oocyte selection occurs exclusively in prefollicular stages [32].

Counting of germ cells in the germinal beds (Fig. **3A**) confirms that cadmium treatment increases the total number of prefollicular oocytes: they reach 760 ± 124 units, value significantly exceeding that measured in November and December controls (342 ± 56 and 319 ± 61 respectively). In estradiol and FSH treated animals the oocytes number also raises significantly reaching 1047 ± 186 and 641 ± 122 units respectively; in progesterone treated animals, by contrast, the number remains at values typical of controls.

Counting of germ cells in the different stages of oogenesis indicates that all treatments significantly change the oocyte hierarchy. Cadmium, in particular, stimulates oocyte recruitment since increases the number of preleptotene oocytes (Fig. **3B**) that rise to 504 ± 125 units, value significantly higher than that registered in November and December controls (231 ± 15 and 197 ± 34 units). The same effect is observed after FSH (430 ± 68 units) but not after estradiol (220 ± 33 units) or progesterone (158 ± 21 units) treatments.

Treatments also affect the number of zygotene-diplotene oocytes (Fig. **3C**). Cadmium in particular, increases their number up to 257 ± 63 units as compared to November and December control ovaries in which 112 ± 25 and 120 ± 24 units are registered, respectively. Zygotene-diplotene oocytes increase in number also after FSH (209 ± 28 units) and estradiol (827 ± 125 units) but not after progesterone (145 ± 23 units) treatment.

Investigations on follicle recruitment (Fig. **3D**) indicate that cadmium does not induce significant changes in the number of primary follicles present in the germinal beds; values, in fact, remain at 2.5 ± 0.4 units, value comparable to that registered in November and December controls (2.5 ± 0.2 and 2.7 ± 0.3 units). By contrast, after estradiol, progesterone and FSH values raise to 6.2 ± 1.2, 4.3 ± 0.9 and 3.8 ± 0.6 units respectively, conditions typical of the Spring recrudescence [31].

DISCUSSION

Cadmium has long been recognized as a cellular toxicant. More recently, it has been recognized also as a potential endocrine disrupter exerting an estrogen-like activity *in vitro* and *in vivo* [9, 34]. In lizard, a single intraperitoneal injection of cadmium results in metal accumulation in various organs, including the ovaries [25], without any effect on animal survival, and in an increased expression of metallothionein [25, 35]. Although this protein protects cells against the toxic effects of cadmium [36,37], the present observations indicate that the ion reaches the germinal beds where stimulates oogonial proliferation and the recruitment of prefollicular oocytes. This data clearly contrasts with what reported, for example, in turtle embryos in which the ion decreases oogonial proliferation [27] or in mammals in which the effects on proliferation of gonocytes (spermatocytes) [38] are not significant.

The effects exerted by cadmium in Podarcis are not typically oestrogenic as expected [34] but FSH-like. The ion, in fact, as the gonadotropin, stimulates the recruitment of new preleptotene oocytes while estradiol has no apparent effect. This result agrees with the evidence that germ cell proliferation in lizards is under the control of FSH [39], while is in contrasts with what reported for other vertebrates in which estradiol and progestins would also play fundamental roles [40, 41].

Results also demonstrate that the supernumerary oocytes formed after cadmium treatment become apoptotic so that the number of primary follicles does not increase. At the

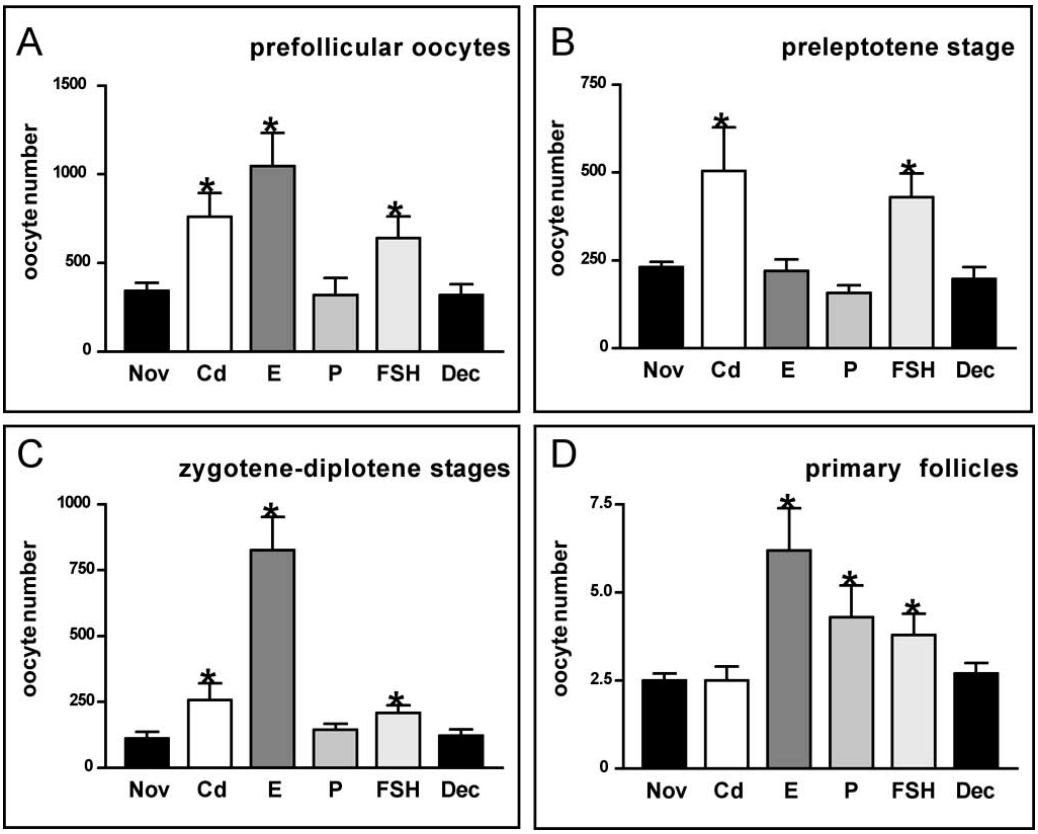

Fig. (3). Changes in oocyte number after cadmium (Cd), estradiol (E), progesterone (P) or follicle stimulating hormone (FSH) treatments. **A**) The total number of prefollicular oocytes increases after cadmium, estradiol and FSH but not after progesterone treatment; **B**) preleptotene oocytes increase in number only after cadmium and FSH treatments; **C**) zygotene-diplotene oocytes increase in number after cadmium, estradiol and progesterone treatments; **D**) primary follicles increase in number after estradiol, progesterone and FSH treatments.
Statistical significancy was assessed by ANOVA. Legend: Nov and Dec, November and December controls; *, significant at $p<0.05$.

moment we do not know the mechanisms triggering such massive degeneration. A first hypothesis is that the formation of extra oocytes would activate the endogenous mechanism controlling germ cell number. In Podarcis this exerts a very strict control since under natural condition the clutch size never exceeds an average of 20 units even though it has been estimated that several thousands new oocytes are recruited every year (unpublished results). This hypothesis is supported by the fact that a similar selection also occurs after treatment with FSH, factor controlling oogonial proliferation and oocyte recruitment [39].

An alternative hypothesis is that cadmium has a direct pro-apoptotic effects on zygotene-diplotene oocytes as reported in other species [27]. Being this true, then the ion in germ cells would exert a two-fold effect: would induce proliferation in oogonial stem cells and death in zygotene-diplotene differentiated oocytes. It is interesting to note that a parallel behaviour is registered in the follicular epithelium in which cadmium induces proliferation in the small stem cells and apoptosis in pyriforms differentiated cells [42]. This evidence is particularly intriguing since suggests that the way of action of cadmium may depend on the stage of the cell cycle. Indeed it has been reported that in Chinese hamster ovary cells the cell cycle progression is retarded as a function of Cd concentration [43].

Another interesting aspect emerging from results is that cadmium induces follicular atresia. In Podarcis, this is a very rare event since oocyte selection occurs between the zygotene stage and the time the primary follicle organizes [32]. The presence of atretic follicles suggests that the metal might reduce fecundity; preliminary data support this conclusion demonstrating a relevant reduction in clutch size.

In summary, our data demonstrate that cadmium in *Podarcis sicula* is an endocrine disruptor capable of stimulating oogonial proliferation and oocyte recruitment by mimicking FSH activity. The ion also exerts toxic effects on the growing follicles thus reducing fecundity and the reproductive performance. The detrimental effects on offspring production may interfere with survival of wild populations inhabiting contaminated areas significantly endangering the local ecological equilibrium.

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Received: October 06, 2009 Revised: January 27, 2010 Accepted: January 27, 2010