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Zinc protection against cadmium-induced infertility in female rats. Effect of zinc and cadmium on the progesterone production of cultured granulosa cells

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Adult female rats were treated subcutaneously (sc) with zinc chloride ($ZnCl_2$, 10 or 20 mg kg^{-1} body weight, bw) four times during two ovarian cycles. The third injection was accompanied by cadmium chloride ($CdCl_2$) administration sc (2.5, 5 and 10 mg kg^{-1} bw). The fourth zinc (Zn) treatment was followed by mating.

$ZnCl_2$ (20 mg kg^{-1}) itself impaired fertility by 20%, while $CdCl_2$ dose-dependently blocked the receptivity of female rats. In combination with 2.5 and 5 mg kg^{-1} $CdCl_2$ the metal salts decreased fertility in an additive fashion, whereas at the highest $CdCl_2$ dose (10 mg kg^{-1}) a marked ameliorating effect of $ZnCl_2$ (10 and 20 mg kg^{-1}) on cadmium (Cd)-caused sterility was observed. In the pregnant animals apart from the higher Cd-induced blood progesterone levels and reduced body weight gain of dams, no significant treatment-related maternal and fetal effects could be observed.

$ZnCl_2$ (10 to 80 μM) and $CdCl_2$ (10 to 80 μM) were added to the culture medium of ovarian granulosa cells. $CdCl_2$ suppressed follicle-stimulating-hormone- (FSH-) and cAMP-stimulated progesterone accumulation. No protective effect of Zn against Cd-induced drop in progesterone production could be seen, while Zn by itself induced a significant increase in FSH-supported progesterone synthesis.

In conclusion, while Zn protected against Cd-induced sterility *in vivo*, it failed to counteract the direct effect of Cd on steroid biosynthesis. The data indicate that Zn protection does not take place at the level of ovary. Moreover, Zn and Cd seem to affect FSH-stimulated progesterone production by different mechanisms.

Keywords: cadmium, progesterone, sterility, zinc–FSH interaction, zinc protection

Introduction

The role of pre-conception exposures to contaminants in unexplained female infertility is not well understood. Body burdens of cadmium (Cd), a highly toxic environmental pollutant, already approach values considered as critical for the functional impairment of the target organs. A 500–2000 μg kg^{-1} tissue Cd content was measured in clinical liver samples of adult human subjects (Iyengar & Iyengar 1994). This can be attributed to the extremely long biological half-time of Cd, ranging from a few years to at least 100 years, calculated from Cd-accumulation in post-mortem human organs and tissues (Sugita & Tsuchiya 1995).

It has been revealed that endogenous female sex hormones may play a role in the higher concentrations of Cd found in the kidneys of female than in male rats Nishiyama *et al.* 1988). In Japan women have been reported to accumulate larger amounts of Cd than men (Sumino *et al.* 1975). Previously we reported that Cd levels in the human ovary increased linearly between 30 and 65 years of age (Varga *et al.* 1993). Cd has been reported to disturb cyclic function at different levels along the hypothalamic–hypophysial–gonadal axis in laboratory

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rodents (Rehm and Waalkes 1988, Paksy *et al.* 1989, Varga and Paksy 1991, Piasek and Laskey 1994, Massanyi *et al.* 1995).

To date no pharmacological agent has been found to be specifically effective in the treatment of Cd intoxication. At the same time, in animal experiments the protective nature of zinc (Zn) against Cd-caused hepatotoxicity (Goering and Klaassen 1984), carcinogenicity (Waalkes *et al.* 1989), testicular damage (Gunn *et al.* 1968) and ovarian lesions (Rehm and Waalkes 1988) has been reported.

Previously we have reported Cd-induced blocking of ovulation in rats (Paksy *et al.* 1989, Varga and Paksy 1991). The present work was undertaken to examine the influence of repeated exposures to Zn on female fertility following a single Cd injection. In a second experiment the *in vitro* interaction between Zn and Cd in influencing progesterone production by cultured granulosa cells obtained from preovulatory ovarian follicles was studied.

Materials and methods

$CdCl_2 \times 2.5\ H_2O$ was purchased from Reanal (Budapest, Hungary), $ZnCl_2$ from Carlo Erba (Milan, Italy), and pentobarbital from Rhone Poulenc (Paris, France). The 1:1 nutrient mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM-Ham's F-12 1:1) was obtained from Sigma (St Louis, MO, USA; Catalogue number: D 8900). Trypan blue (0.4%) was purchased from Sigma Aldrich Co. (UK), fetal calf serum (FCS) from Humán (Budapest, Hungary). Radioimmunoassay (RIA) progesterone antiserum was kindly provided by Dr G.D. Niswender (Fort Collins, CO, USA). The 24-well culture plates (TC sterile no. 662 160) were purchased from Greiner GmbH (Kremsmünster, Austria).

Animals

Adult virgin female and male CFY rats (LATI, Gödöllő, Hungary) weighing 200–250 g were maintained under controlled light conditions (12 h light, 12 h dark) with free access to rat chow (LATI, Gödöllő, Hungary) and tap water. Ovarian cycle was checked daily by vaginal cytology. Animals displaying at least three 4-day cycles were selected for the experiment.

Statistics

Analysis of variance and Dunnett's test were used for statistical evaluation.

Experimental protocol (Figure 1)

Female rats (10–15 animals per dose) received 10 or 20 mg kg^{-1} bw $ZnCl_2$ injection sc four times during two consecutive ovarian cycles, always at 9.00 pm on estrus and diestrus 2. The third treatment (80 h prior mating on estrus) was accompanied by a simultaneous injection of $CdCl_2$ sc, followed by the fourth (last) $ZnCl_2$ administration. The doses of $CdCl_2$ were 2.5, 5 and 10 mg kg^{-1} bw. The metal doses refer to the salt and not the ion concentrations. Some groups were treated with the two doses of $ZnCl_2$ or three doses of $CdCl_2$ alone. Control rats received 0.9% NaCl solution. On the following proestrus, females were caged with fertile males from 4.00 pm on and overnight. Next morning vaginal smears were taken to check cytology; females having sperms in their smears were designated as Day 1 pregnant animals. Pregnant animals were housed individually. Maternal body weights were measured on the day of treatment and on Days 1 and 12 of pregnancy. On the 12th day of gestation (20 days after the first $ZnCl_2$ and 16 days after the $CdCl_2$ injection) rats were sacrificed under pentobarbital narcosis (40 mg kg^{-1}). Following laparotomy, internal organs were examined and uterine horns excised, weighed and the fetuses counted. The weight of concepti (weight of pregnant uterus per number of fetuses) was recorded. Ovaries were removed, trimmed, then separated into two parts: luteal tissue, i.e. corpora lutea (CL) of pregnancy, and nonluteal tissue, i.e. small follicles and stroma. CL were counted and the wet weight of luteal and nonluteal tissue was measured. Blood samples were taken from the maternal aorta, kept at 4ºC for 24 h and centrifuged; sera were then stored at –20ºC until assayed for progesterone and 17β-estradiol by RIA.

Type of Treatment	Days of Cycle					Duration of Pregnancy
	E	D_2	E	D_2	P	Day 1–Day 12
1.	Zn	Zn	Zn	Zn	♀♂	No
2.	–	–	Cd	–	♀♂	
3.	Zn	Zn	Zn+Cd	Zn	♀♂	Treatment

Figure 1. Treatment protocol for studying $ZnCl_2$ and $CdCl_2$ interaction in Cd-induced antifertility effect in the female rat. $ZnCl_2$ (10 or 20 mg kg^{-1} bw) and $CdCl_2$ (2.5, 5 and 10 mg kg^{-1} bw) was given sc alone or simultaneously (three types of treatments) on selected days of the cycle, followed by mating. Control groups received 1 ml kg^{-1} bw 0.9% NaCl solution. (E = estrus; D_2 = diestrus 2; P = proestrus; σσ = mating.

Isolation and treatment of granulosa cells

Animals were anesthetized (40 mg kg^{-1} pentobarbital) on proestrus at 8.00–9.00 am. Uterine ballooning was checked, and ovaries were removed and cleaned. The largest (0.6–1.0 mm in diameter) – and due to starting vascularization – slightly reddish preovulatory follicles were selected, counted and punctured by a 26½ gauge stainless steel needle. Granulosa cells were released into the chilled medium (DMEM-Ham's F-12 1:1 containing

10% FCS) using gentle pressure and agitation of the theca capsule. To disintegrate cell clumps the preparation was aspirated vigorously with an automatic pipette holding a sterilized plastic tip. Cells were counted and diluted with the culture medium to a final concentration of 3×10^5 cells per ml per well. Viability was 80–85% as estimated by the Trypan blue exclusion test. The cell suspension was dispersed to 24-well culture plates.

Cells were incubated in the presence or absence of FSH (100 ng ml^{-1}) and cAMP (1 μM) to control secretagogue-induced response of cells, and exposed to 0–50 μM $CdCl_2$ to find the effective concentration range. In a separate experiment single or combined exposures to $ZnCl_2$ (10–80 μM) and $CdCl_2$ (10–80 μM) were made. Incubations were carried out in duplicate for 48 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. At the end of exposure, spent media were collected, centrifuged ($900 \times g$, 10 min, 4°C) and frozen (–20°C) for later progesterone analysis. FSH, cAMP and metal salts were dissolved in medium without FCS. Experiments were repeated at least three times (five or six rats, 8–10 follicles per rat, i.e. 40–60 follicles per experiment).

Results

Fertility study (Figure 2)

20 days after the first $ZnCl_2$ and 16 days after the $CdCl_2$ injection all animals survived. While $CdCl_2$ dose-dependently decreased, $ZnCl_2$ did not markedly influence the number of mating female rats compared to control animals.

As a result of simultaneous treatment at $CdCl_2$ doses of 2.5 and 5 mg kg^{-1}, $ZnCl_2$ (20 mg kg^{-1}) + $CdCl_2$ treatment significantly decreased mating rate compared to controls (significance is designated by *). In rats receiving 10 mg kg^{-1} $CdCl_2$ the Cd-induced 100% lack of receptivity could be reversed by Zn in a dose-related fashion.

The nearly 60% receptivity of rats treated with $ZnCl_2$ (20 mg kg^{-1}) + $CdCl_2$ (10 mgkg^{-1}) proved to be statistically significant when compared to the mating rate of animals treated with $CdCl_2$ (significance is designated by the letter b).

In successfully mated rats receiving 10 mg kg^{-1} $CdCl_2$ and 10 mg kg^{-1} $ZnCl_2$ a significant reduction in maternal body weight gains and a marked elevation in aorta progesterone levels was observed. Weight increment was calculated from the day of Cd treatment (estrus) until Day 12 of pregnancy (16 days). No statistically significant alterations of E_2 levels in maternal blood, weight of concepti (Table 1), number and weight of corpora lutea, or fetal numbers (data not shown) could be recorded. In nonpregnant rats ballooned uteri were found in 30% of animals.

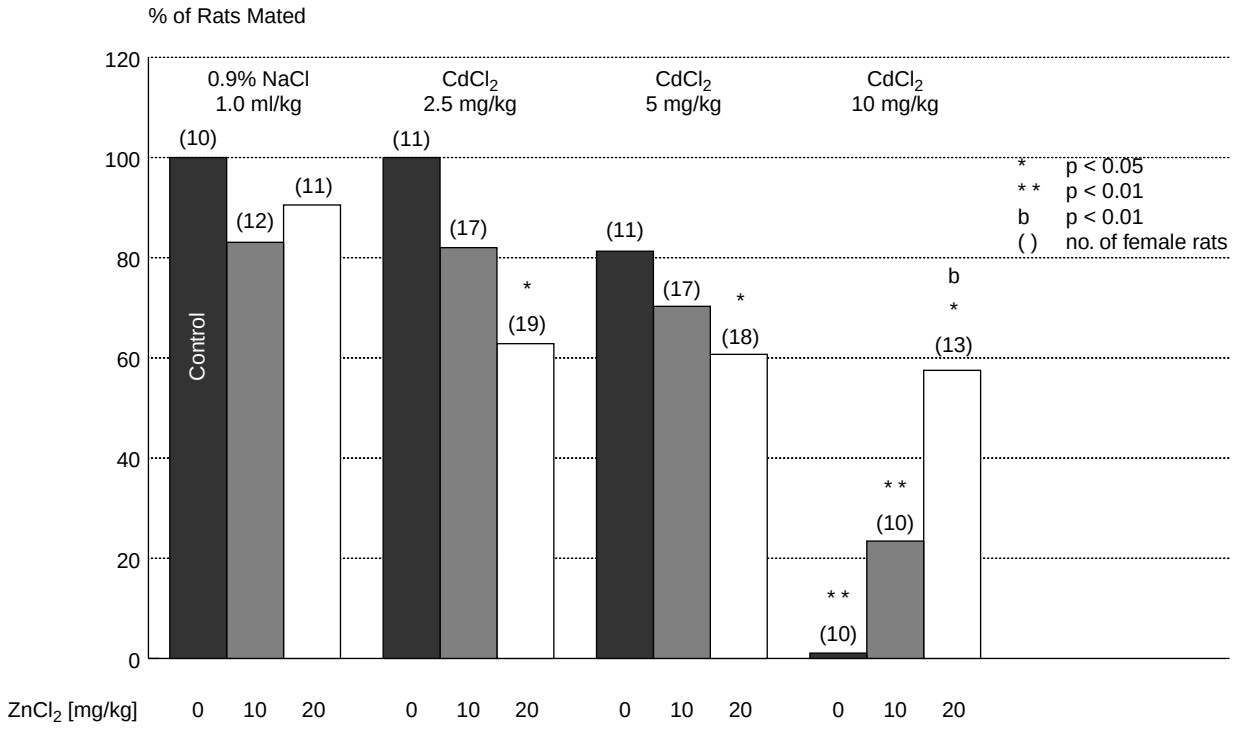

Figure 2. Effect of separate and combined treatment with $ZnCl_2$ and $CdCl_2$ on the mating rate of female rats. *, ** = comparisons with the controls; b = comparisons between Cd and Cd + Zn treatments.

Table 1. Maternal and fetal effects of pre-conception separate and concomitant subcutaneous $ZnCl_2$ and $CdCl_2$ administrations on the twelfth gestational day in rats. Treatment protocol is demonstrated in Figure 1

$CdCl_2$ (mg kg^{-1})	$ZnCl_2$ (mg kg^{-1})	Progesterone (ng ml^{-1})	Estradiol (pg ml^{-1})	Maternal bw increment (g)	Weight of concepti (mg)
0.9% NaCl	0	28.69 ± 2.48 (10)	72.33 ± 3.7 (10)	70.0 ± 4.9 (10)	265.4 ± 9.1 (10)
	10	33.23 ± 3.16 (8)	–	66.3 ± 4.1 (10)	259.8 ± 13.4 (10)
	20	29.59 ± 2.8 (9)	67.65 ± 3.70 (7)	71.5 ± 6.9 (10)	272.1 ± 11.1 (10)
2.5	0	33.32 ± 2.65 (9)	67.55 ± 5.90 (10)	69.9 ± 5.2 (11)	251.1 ± 10.9 (11)
	10	34.41 ± 3.31 (14)	–	64.1 ± 4.6 (15)	255.3 ± 6.3 (14)
	20	32.50 ± 3.31 (11)	61.26 ± 4.35 (8)	77.2 ± 3.4 (12)	266.2 ± 7.9 (12)
5	0	29.14 ± 2.41 (7)	63.36 ± 2.65 (7)	70.6 ± 5.2 (9)	292.2 ± 6.4 (9)
	10	35.61 ± 2.04 (10)	–	57.3 ± 5.1 (13)	247.6 ± 6.4 (12)
	20	27.12 ± 4.15 (10)	68.16 ± 4.45 (10)	75.6 ± 4.9 (11)	266.1 ± 9.7 (11)
10	0**	–	–	–	–
	10	55.68* ± 6.06 (4)	–	41.7* ± 2.3 (7)	238.0 ± 17.4 (4)
	20	32.33 ± 3.07 (11)	67.46 ± 4.06 (11)	70.5 ± 5.2 (11)	259.0 ± 10.7 (11)

* $P < 0.01$ (compared to the control).
**10 mg kg^{-1} $CdCl_2$ induced 100% sterility.
± SE
( ) *n*

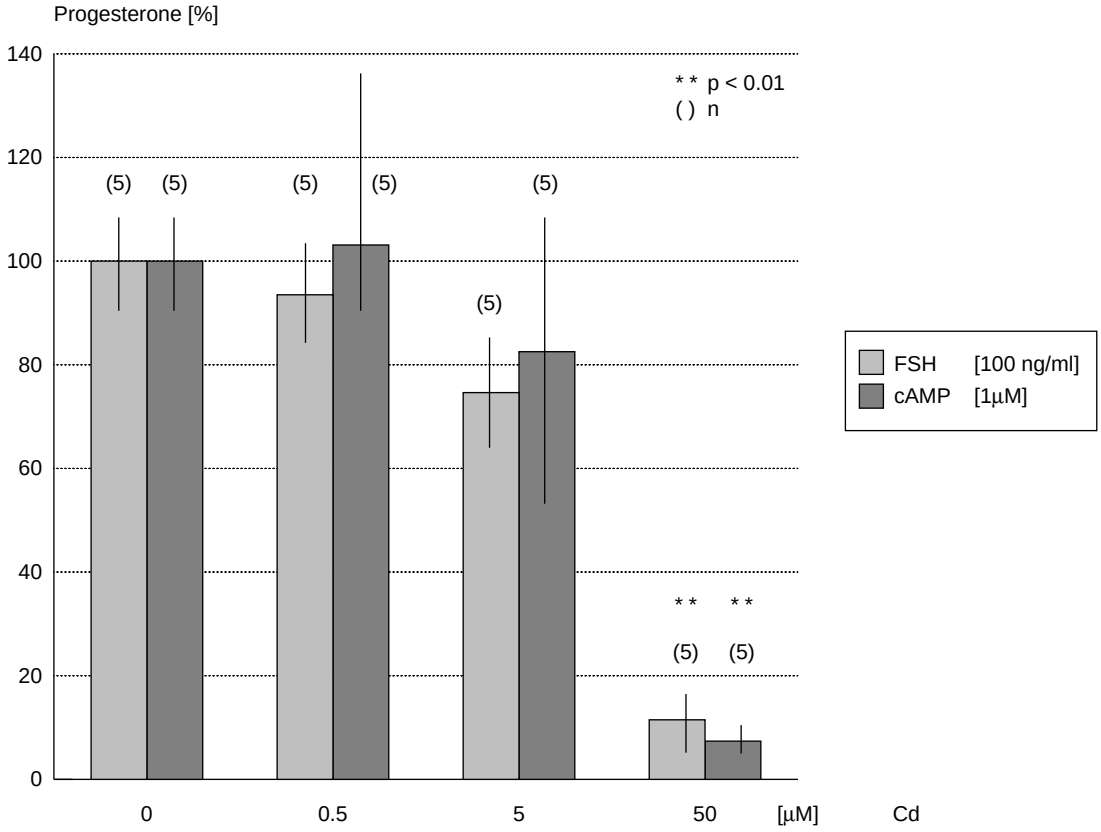

Figure 3. Effect of $CdCl_2$ on FSH- and cAMP-induced progesterone accumulation *in vitro* on granulosa cells obtained from antral follicles of proestrus rats. Data are expressed as percent of FSH- and cAMP-induced progesterone production of 3×10^5 cells over 48 h (19.17 ± 2.6 ng ml^{-1} and 16.93 ± 2.88 ng ml^{-1}, respectively = 100%, n = 5); basal progesterone production = 3.39 ± 2.04 ng ml^{-1}, n = 18. Progesterone synthesis of stimulated cells is compared to that of Cd-exposed cells.

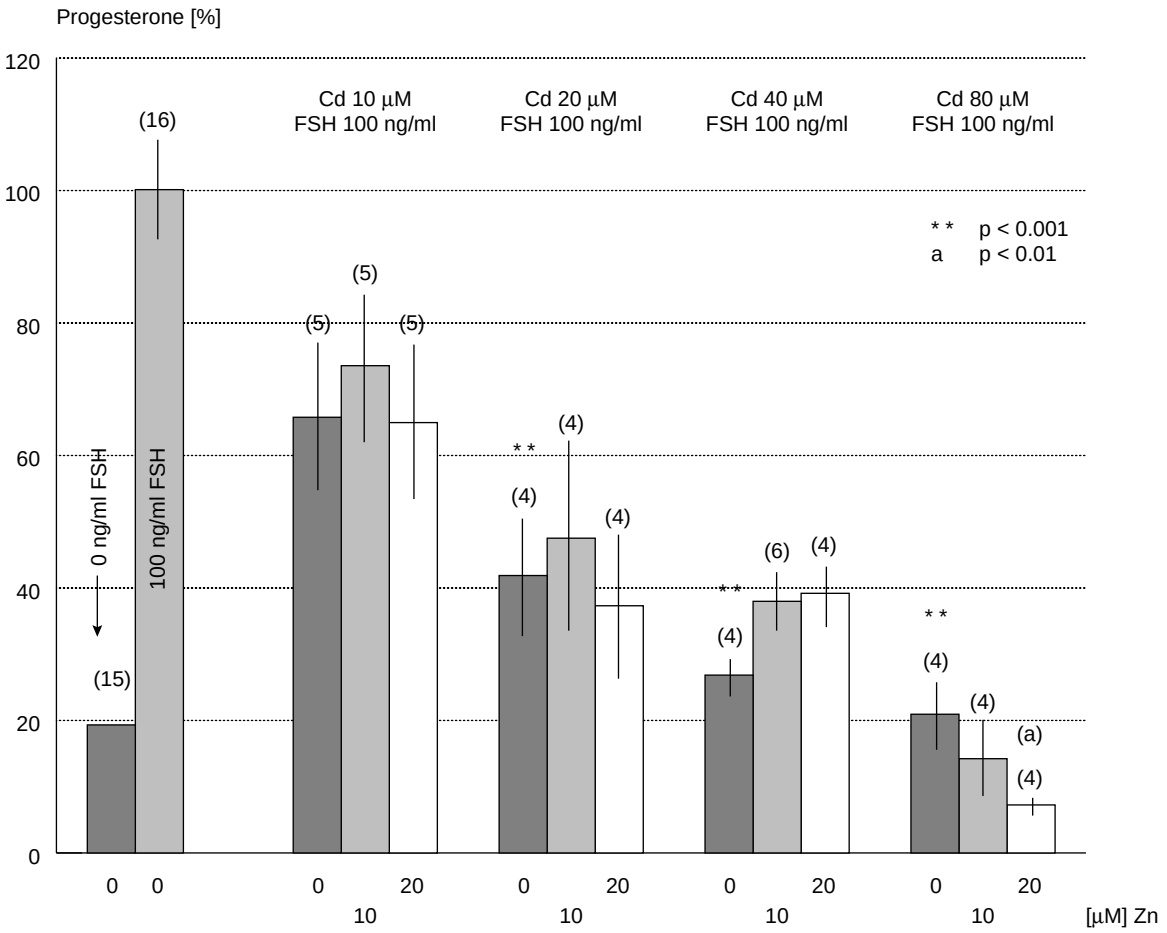

Figure 4. Effects of separate and combined exposure to $CdCl_2$ and $ZnCl_2$ on FSH-stimulated progesterone production of cultured granulosa cells obtained from antral follicles of proestrus rats. Data are expressed as percent of FSH-induced progesterone production of 3×10^5 cells over 48 h. The first two columns represent the baseline (3.32 ± 1.34 ng ml^{-1}) and FSH-stimulated (19.17 ± 2.6 ng ml^{-1} = 100%) progesterone accumulation of unexposed cells. Cd and Zn exposures were carried out in the presence of 100 ng ml^{-1} FSH. ** = comparisons between control and $CdCl_2$-exposed cells; a = comparisons between $CdCl_2$- and $CdCl_2$ + $ZnCl_2$-exposed cells; ( ) = number of experiments.

Progesterone accumulation in granulosa cell cultures

Progesterone production of unstimulated cells during the 48 h incubation period was 3.39 ± 2.04 ng ml^{-1} (n = 18) while 100 ng ml^{-1} FSH- or 1 μM cAMP-stimulated progesterone accumulation was as high as 19.17 ± 2.6 ng ml^{-1}, n = 5, and 16.93 ± 2.88 ng ml^{-1}, n = 5, respectively. Experiments were always carried out on stimulated cells. The stimulated levels were considered as 100%. Cd suppressed both FSH- or cAMP-supported progesterone accumulation in a concentration-dependent manner (Figure 3).

Cd induced significant, dose-dependent reduction in FSH-stimulated granulosa cells compared to FSH-stimulated controls (Figure 4, symbol **) and this effect could not be markedly counteracted by Zn applied in equimolar quantity with the lower Cd concentrations. On their own, Zn exposures to higher concentrations than 80 μM blocked FSH-simulated steroidogenesis (data not shown).

The difference between the slightly higher progesterone values measured in Cd + Zn + FSH-exposed cultures compared to the values in Cd + FSH-exposed ones was not statistically significant; moreover, the combined treatment at doses of 80 μM Cd and 20 μM Zn resulted in a marked decrease in FSH-stimulated progesterone synthesis compared to separate exposure to Cd (Figure 4, letter a). Zn given to cell cultures for 24 h, as pretreatment, was also unable to prevent Cd toxicity (data not shown).

Interestingly enough, at the same time in cells exposed to Zn and FSH without Cd – serving as control exposure to Zn + Cd + FSH-exposed cells – a significant increase in progesterone production was found. Thus, Zn potentiated FSH-stimulated progesterone biosynthesis at concentrations of 10–20 μM, with a maximum at 20 μM, compared to progesterone accumulation when cells were stimulated with FSH alone. At Zn concentrations as high as, and higher than 40 μM, the FSH-stimulated progesterone accumulation decreased (Figure 5).

Discussion

In studies in mature rats (Rehm and Waalkes 1988) $CdCl_2$ did not induce ovarian lesions at 20 μM kg^{-1} (4.6 mg kg^{-1}) $CdCl_2$ dosage. At 40 μM kg^{-1} (10.93 mg kg^{-1}) ovarian follicular necrosis varied, affecting 5–50% of the follicles in section. No renal or pancreatic lesions were observed in any of the rats treated with 4.6–10.93 mg kg^{-1} $CdCl_2$. The results of another histopathologic evaluation revealed no Cd-related alterations in the liver, kidney or uterus of cycling and pregnant rats, nor in the ovaries of proestrus animals at doses of 3 or 5 mg Cd kg^{-1} bw as $CdCl_2$ (Piasek and Laskey 1994).

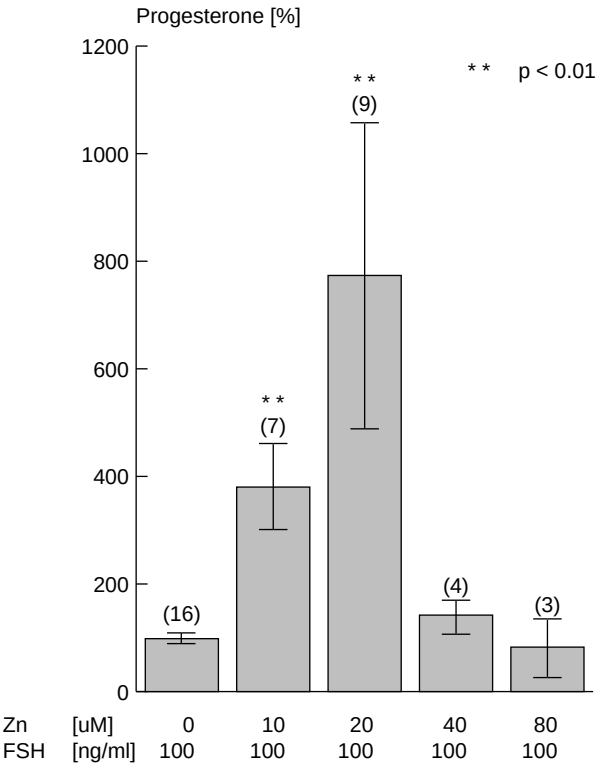

Figure 5. Interaction between $ZnCl_2$ and FSH, influencing progesterone production of cultured granulosa cells. Data are expressed as percent of FSH-induced progesterone production of 3×10^5 cells (19.17 ± 2.6 ng ml^{-1} = 100%) over 48 h. Comparisons are made between the stimulated and the stimulated + $ZnCl_2$-exposed cells. ( ) = number of experiments.

In the CFY rats used in our present and earlier (Paksy *et al.* 1989) studies, no Cd-induced ovarian histopathologic changes were found; at the same time the number of mating female rats dose-dependently decreased. This phenomenon is in good accordance with our previous findings obtained with similar doses; Cd induced anovulation, checked by counting ova shed (Paksy *et al.* 1989). Cd-induced block has been suggested to be caused: (1) by disturbed function of pituitary; (2) via interference with ovarian steroid secretion.

(**1**) In earlier reports we have shown that following Cd administration, Cd accumulated in the pituitary; the luteinizing hormone (LH) content in the tissue of pituitary decreased, while the weight of pituitary remained unchanged; on the afternoon of proestrus blood LH levels were significantly lower than required for the LH surge and ovulation; this Cd-induced anovulation could be *restored* by excess dose of the hypothalamic luteinizing hormone releasing hormone (LHRH) (Paksy *et al.* 1990, Varga and Paksy 1991). Further confirmation of our findings is that Cd has been reported to block Ca-triggered LH-release in sheep pituitary cells by directly inhibiting exocytosis at an intracellular site (Davidson *et al.* 1993).

(**2**) Cd has also been found to directly influence ovarian function by decreasing ovarian secretion rate of progesterone in rats (Paksy *et al.* 1989). Increased progesterone secretion on the afternoon of proestrus plays an important role in amplifying and synchronizing LH release by enhancing the activation of LHRH neurons (Lee *et al.* 1990).

The above evidence, along with the findings of the present study that Cd decreases the mating rate of female rats, demonstrates that Cd exerts a *specific* effect on the interdependent *regulatory* mechanism of gonadal function in the adult female rat.

Further effects of Cd

Increased blood progesterone levels in Cd-treated rats may be due to Cd-induced decreased activity of hepatic cytochrome P450 enzymes that take part in progesterone metabolism (Unger and Clausen 1973).

Possible mechanism and site of Zn protection

In this study Zn given as pretreatment was able to dose-dependently counteract the single-dose Cd-induced sterility. It is hypothesized that there may be more avenues for Zn to counteract this rather complex toxic symptom. It has been proposed that Cd uses the transport pathways that exist for essential metals. Zn has been reported to antagonize cellular Cd transport and accumulation by competing for binding to sulfhydryl ligands (Shaikh *et al.* 1995). Cd has been revealed to mobilize cell calcium and Zn was able to selectively and reversibly block this process (Smith *et al.* 1989). The role of calcium entry and intracellular calcium in coordinating regulation of hormone secretion is well recognized (reviewed by Kiesel 1993). Since at the low Zn dose certain symptoms of overt toxicity such as reduced maternal body weight gain or raised blood progesterone levels remained, and at the high Zn dose no symptoms of toxicity were observed, an indirect method of Zn protection against Cd-induced infertility can also be suggested. Evidence is accumulating about the role of the metal-binding protein metallothionein (Mt) in the development of tolerance to Cd toxicity. This is a low molecular weight protein which shows high affinity for bivalent cations such as Zn^{2+} and Cd^{2+}, and which can be induced in several organs, mainly the liver and kidneys (Muller *et al.* 1988). In rodents Cd has been described as entering the circulation slowly from the site of a subcutaneous injection. Blood levels elevate for 2 days in rats (Paksy *et al.* 1990). After 4–7 days 7–15% of the doses (2.2–4.4 mg kg^{-1}) could still be found there, serving as a pool (Kasparzak and Poirier 1985). Soon after, the superinduction of hepatic Mt mRNA occurs because of the continued influx of Cd over 5–10 days. Mt is able to reduce the bioavailability of highly toxic free Cd^{2+} and Cd bound to albumine and mercaptalbumine with low stability. It is known, however, that *de novo* Mt biosynthesis is time-dependent, taking 16–32 h (Scheuhammer *et al.* 1985), and Cd ions not bound to Mt may disrupt normal cell processes *prior* to renal induction of Mt (Li *et al.* 1993). Nevertheless, if repeated Zn pretreatments take place inducing Mt biosynthesis, pre-existing Mt is supposed to immediately sequester the toxic Cd species, preventing its interaction with susceptible subcellular organelles (Goering and Klaassen 1984). Mt inducibility by Cd has also been reported in pituitary cells (Hinkle *et al.* 1987); at the same time Zn, like Cd, has also been shown to inhibit Ca-triggered LH release in gonadotrophs (Davidson *et al.* 1993).

To the best of our knowledge, no data on Mt content of the rat ovary have been reported. The hamster ovary has shown to be deficient in Mt (Waalkes *et al.* 1988). Our present *in vitro* findings have also failed to support a theory that Zn protection takes place at the level of ovary via counteracting Cd-induced disturbance of progesterone biosynthesis in granulosa cells.

Possible mechanism of Cd effect on steroidogenesis in vitro

Progesterone production of granulosa cells depends on the level of cAMP, which is the second messenger in the signal transduction in FSH-stimulated steroidogenesis. In our study Cd reduced progesterone production in FSH- as well as cAMP-facilitated cells. It is postulated, therefore, that Cd may affect steps of signal transduction following the second transmission, cAMP accumulation and/or degradation. This theory is supported by the finding that phosphodiesterase activity is enhanced by Cd via calmodulin-dependent mechanisms (Suzuki *et al.* 1985), which can lead to decreased cAMP accumulation and a subsequent lowering of progesterone production. This is in accordance with the findings of Laskey and Phelps (1991) who reported decreased testosterone production in hCG- or cAMP-facilitated cultured mouse Leydig cells exposed to Cd. These authors suggest that the inhibiting site(s) is (are) beyond the membrane receptor and before the mitochondria.

These *in vitro* findings confirm our earlier *in vivo* results, which have demonstrated that Cd decreases basal and human chorion gonadotropin (hCG)-stimulated progesterone secretion rate measured in ovarian venous blood of estrus rats 2 days after subcutaneous treatment (Paksy *et al.* 1989).

Interaction between Zn and FSH

A significant finding of this study seems to be that Zn exerts a dual effect, stimulating FSH-facilitated progesterone biosynthesis in the range of 10–20 μM and decreasing it at concentrations higher than 40 μM. To our knowledge, no similar effects on rat granulosa cells have been reported so far.

Zn has been reported to increase spontaneous catecholamine release and facilitate evoked secretion in bovine chromaffine cells at 3 μM and inhibit it at 100 μM (Vega *et al.* 1994); these concentrations and effects are similar to those found in our study. These data and our finding of the dual effect of Zn are in good accordance with the results and

conclusions of Heng and coworkers (1993), who reported that intradermal Zn injections brought about significant elevation of cAMP levels in the cytosol of epidermal cells of nude mice. It has been reported that Zn is able to compete for calcium binding sites on the plasma membrane. Calcium activates calmodulin, which stimulates the activity of both the adenylate cyclase and nucleotide phosphodiesterase. Thus, inhibition of the calcium-activated calmodulin complex by Zn may either increase or decrease intracellular cAMP levels depending on the relative interaction between its synthetic and degradation enzymes.

Lack of Zn protection in cultured granulosa cells

In the present study Zn itself exerted a toxic effect on steroidogenesis in ovarian granulosa cells at concentrations as high as, and higher than 40 μM. Lower concentrations did not influence this process; however, they did not antagonize Cd-evoked reduction in progesterone accumulation. The protective role of Zn in various *in vitro* systems seems not to be unequivocal. In cultured rat primary hepatocytes (Liu *et al.* 1990) and in monolayer culture of bovine aortic endothelial cells (Kaji *et al.* 1993) Zn protected against Cd toxicity; by contrast, in renal proximal tubular cells (LLC-PK cells) toxicity due to Cd and Zn is not affected by the simultaneous administration of the other metal (Harford and Sarkar 1991).

In the present study Zn protection could have been expected based on its proposed role as a direct Mt-inducer, evoking Mt synthesis *in vivo* as well as *in vitro*. Further studies are needed to confirm whether the failure of *in vitro* protection may be due to deficiency of inducible Mt in ovarian granulosa cells of the rat such as has been reported in the ovary of Syrian hamster (Waalkes *et al.* 1988).

Calmodulin is known to play a key regulatory role in FSH-facilitated steroid secretion in ovarian granulosa cells (Tsang and Carnegie 1983). A possible explanation for the failure of Zn protection in our study may be the ability of both metals to exert a nearly similar effect on calmodulin, the intracellular calcium modulator. Both Zn and Cd induce a similar large conformational change in the protein, resembling that induced by calcium. It seems that the presence of Zn, despite its having a lower binding capacity to calcium binding sites than Cd – which binds to all calcium binding sites of calmodulin (Sutoo *et al.* 1988) – has not influenced calcium activation of calmodulin in a positive way in terms of counteracting Cd effects on FSH-stimulated steroidogenesis. Further studies are required to determine the exact role of Zn, and the site of action for its protective effect against Cd-induced sterility.

Conclusion

Cd was shown to be a potent antifertility agent in female rats. It reduced steroidogenesis in cultured granulosa cells at a concentration one order of magnitude higher (20 μM e.g. 2.3 μg Cd ml^{-1}) than the highest Cd levels (0.512 $\mu g g^{-1}$ wet weight, mean: 0.149 ± 0.020 $\mu g g^{-1}$) measured in the ovarian tissue of women smokers in our earlier study (Varga *et al.* 1993).

While Zn protected against Cd-induced sterility *in vivo*, it failed to counteract the direct effect of Cd on steroid biosynthesis *in vitro*. Interestingly, Zn potentiated FSH-stimulated progesterone production, but failed to antagonize markedly Cd effects on FSH-stimulated progesterone accumulation in cultured granulosa cells.

It is proposed that *in vivo* Zn protection does not take place at the level of the ovary; moreover Zn and Cd seem to affect FSH-stimulated progesterone production by different mechanisms.

It is known that progression and outcome of pregnancy is dependent upon Zn, and that congenital malformations result from low intakes of Zn (Morgan *et al.* 1995). Considering the findings of the present study, Zn status seems to play an important role in protecting against possible exposure to one of the most toxic environmental pollutants, cadmium, preceding conception as well.

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