厚生労働科学研究費補助金

(化学物質リスク研究事業)

胎生期の水銀およびカドミウム曝露による神経行動毒性の
高感受性群におけるリスク評価に関する研究

平成 16 年度　総括・分担研究報告書

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平成 17 年（2005 年）4 月

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厚生労働科学研究費補助金（化学物質リスク研究事業）

総括研究報告

周生期カドミウム曝露が出生後の神経行動機能におよぼす影響

－加齢後における評価－

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研究要旨

要旨：周生期カドミウム（Cd）曝露が，出生後の神経行動機能に影響をおよぼすことを前年度報告した．本年度は，影響の見られたコホートを用い，15 ヶ月齢において行動試験を実施し，影響の可逆性について検討した．その結果，前年度と同様のパラメタを用いて行った受動回避試験において，オスでは系統にかかわらず，Cd 曝露群のみに学習効果の認められた個体が存在した．オープンフィールド試験では，野生型オスにおいて，Cd 曝露群が非曝露群に比較して移動量が大きい傾向があったが,有意差には至らなかった．

　これらの結果は，前年度報告した行動影響は約1年後には統計的には認められなくなっていることを示したが，前年度影響が認められたメス（オスでは影響を認めなかった）についての検討ができなかったこと，オスでも有意差には至らないものの Cd の影響をうかがわせる結果があったことなどを考慮すると，行動試験について naïve なマウスを用いて加齢後の試験を行う必要があるものと思われた．

はじめに

前年度報告において，周生期の Cd 曝露によって出生後の行動機能に影響が検出されることを示した．発達神経毒性はしばしば不可逆的であり，Cd による毒性についてもその可逆性を検証することが重要である．そこで，昨年度生後 9-10 週齢において行動機能に供したマウスを用通常餌で飼育の後，14 ヶ月に達した時点で再び行動試験に供し,周生期における Cd 曝露の影響の持続性を

検討した.

方法

使用動物, 曝露方法, 行動試験の方法などは前年度の報告と同様である. 簡単に記すと, C57BL/6系（野生型；wt）およびそのメタロチオネインノックアウト（MT-null）系のマウスを交配, プラグ確認した妊娠個体に, プラグ確認日より Cd10ppm を添加した milliQ あるいは非添加 milliQ を与えた. 出産後も授乳 10 日目（仔にとっての生後 10 日目；PND10. なお, 出生を確認した日を PND0 とした）まで Cd 添加／非添加水による飼育を続け, PND11 以降はすべて非添加の milliQ に切り替え, PND28 に離乳, 以降は雌雄別に飼育を続けた. なお, リッターサイズの影響を避けるため, PND 2 において, リッターサイズを 6 に揃えた.

行動試験は, 各リッターより雌雄各 1 匹ずつをランダム選び, すべての行動試験において同一の個体を繰り返して用いた.

8-9 週齢において情動性／活動性の評価法であるオープンフィールド（OPF）, 学習評価法である受動回避試験（Passive Avoidance；PA）を, ここでメスのみに影響が認められたことを踏まえて, 約 4 ヶ月齢においてメスのみを対象に, 空間学習評価法である放射状迷路（Radial Maze）を実施した. これらの結果はすでに前年度の報告したとおりであり, PA においては, メスのみで Cd 曝露群の学習成績が非曝露群よりも劣っており, MT-null 群でその差がより顕著であった. オスではこのような差を認めなかった. Radial Maze（前述のようにメスのみを用いて実施）においては, 迷路学習の獲得（acquisition）においては, 系統差・曝露効果ともに認めなかったが, 学習を獲得した後, 課題の難易度を上げるプロセスを導入したところ, MT-null において, Cd 曝露群の成績が非曝露群に比べて劣る傾向を示した. OPF においては, Cd の影響を検出できなかった.

今年度は, 同じ OPF, PA の試験装置を用い, 15 ヶ月齢で試験を実施した結果を報告する.

結果

メスでは, Radial Maze に供した際に, 絶食をかける必要があった（オスでは Radial Maze は実施していない）が, 予備的な検討に基づいて絶食を行ったにもかかわらず一部の動物が死亡し, 結果として wt の非曝露群が残らなかった. 生存した個体でも絶食の影響が無視し得ない可能性があるため, メスについては加齢期のデータを用いないこととした. 以下に述べる結果は, 全てオス（n=4-8／群）についての結果である.

OPF：図 1 に結果を示した. wt において, Cd 曝露群が対照群の 1.5 倍の移動量を示したが, 統計的に有意な差には至らなかった. MT-null ではこのような差を認めなかった.

OPF of aged Cd-exposed mice
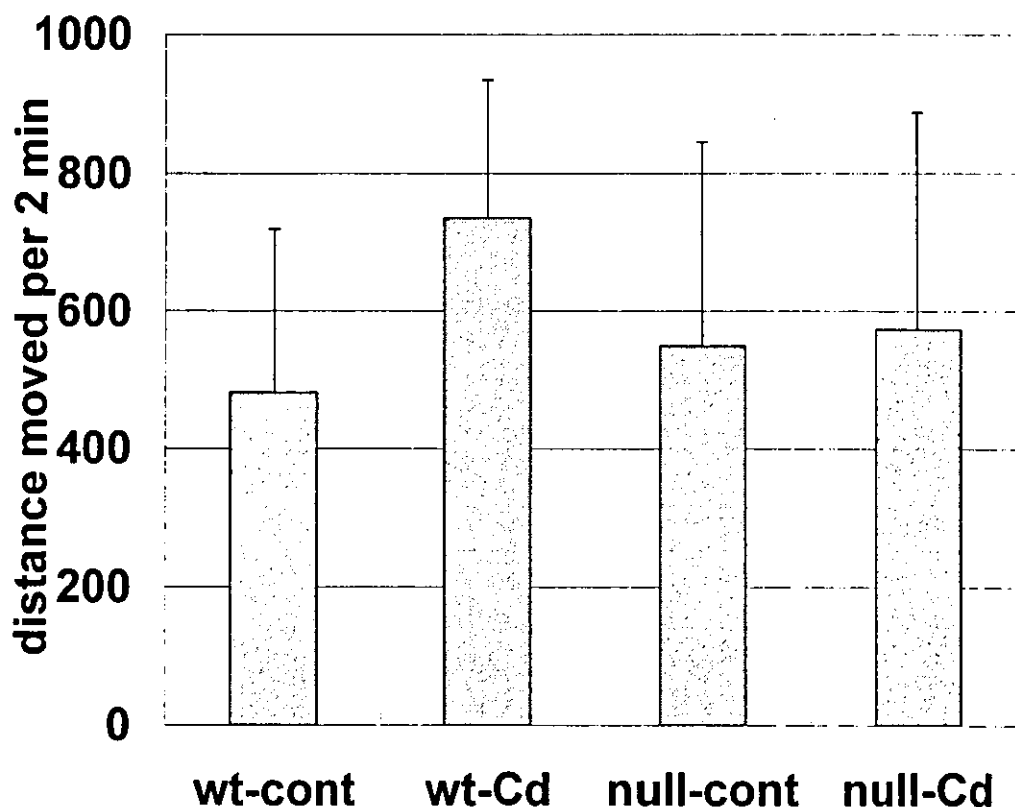
図1．周生期 Cd 曝露♂マウスの加齢後（15mos）におけるオープンフィールド試験
での移動量（縦軸：2分間の移動量[cm]）

PA：図2に結果を示した．図において，latency は明室におかれたマウスが暗室に移動するまで
の時間を秒で表している．training（訓練試行）は第1日目（学習成立前）の試行で Cd 曝露群で
平均値がやや大きい傾向があるが，有意差には至らなかった．暗室にはいったマウスは，その直
後に短い微弱電流でショックを受け，嫌悪学習が成立する．Retention（保持試行）は Training
の翌日に実施し，学習が成立していれば暗室にはいるまでの時間が長くなることが想定されてい
る．実際には wt では学習が成立していない可能性が高い．一方 MT-null では latency が伸びる
（学習が成立していることに相当）傾向が見られ，Cd 曝露群でその傾向が強かった．ただし，
個体間のバラツキは大きく，Cd・strain ともに統計的には有意な効果を検出できなかった．

Passive Avoidance
15mos-old males perinatal exposure to Cd
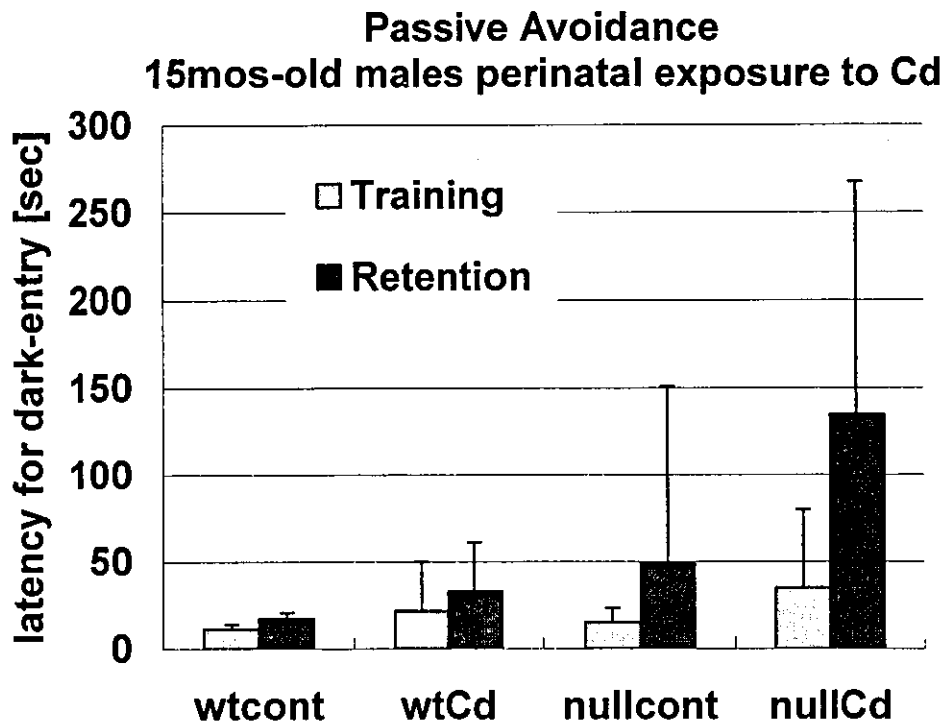
図2．周生期 Cd 曝露♂マウスの加齢後（15mos）における受動回避試験（PA）（縦軸：暗所へ移動するまでの時間［秒］）

考察

統計的には，周生期の Cd 曝露が出生後1年以上経過したマウスの行動に及ぼす影響を確認することができなかった．ただし，出生後3ヶ月齢（OPF, PA）あるいは6ヶ月齢（RM）で影響が認められたのはメスであったが，今回はメスについて検討を実施できていないことから，周生期 Cd の行動影響が可逆的であると結論するには，この結果は不十分である．本研究の期間終了後にはなるが，現在新たに Cd 曝露のコホートを維持しており，このコホートを用いて加齢後の再評価を実施する予定である．

オスについて，OPF では統計的な有意差を認めなかったが，wt では Cd 曝露による活動昂進の傾向が認められ，個体毎のデータ（本書には示さず）を見ても，両群の差が示唆される．現在維持中のさらにn数の多いコホートでこれらの結果を追試する予定である．
　　PA については野生群において学習が成立していない．本試験での実験パラメタは前年度と同一であり，前年度オスでは系統・処理とは関わりなく，学習が成立した（retention trial での latency が長かった）ことから考えると，今年度の結果は加齢による学習能の変化を示唆している．ただ

し，これが移動-刺激という関連を学習する能力，嫌悪刺激の記憶保持能力，あるいは電流刺激への感受性のいずれが低下したことによるものなのかは不明である．今後の検討課題であろう．

　統計的には有意差に至らなかったが，wt と比較した場合，MT-null，特に Cd 曝露群では retention time が長い傾向が見られた．この点も現在維持中のコホートの加齢後に追試する予定であるが，PA における嫌悪学習能力が見かけ上，向上したことについては，上記にあげた以外にも不安のレベル，活動性のレベルなど，学習能以外の要素も影響を与えているものと思われる．本研究の範囲では，系統差を示す可能性があることと，MT-null において，Cd 曝露が何らかの影響を残している可能性があることの 2 点を指摘しておきたい．

　なお，結果には示さなかったが，本年度は，周生期 Cd 曝露の新たなコホートを作製し，生後 3 ヶ月齢における行動影響の追試を行った．結果は，OPF において，メスで系統によらず Cd 曝露群の移動量が低下していた（2-way ANOVA），オスでも曝露による活動抑制傾向はあったが，有意ではなかった．PA においても，メスのみにおいて training trial の latency が曝露群＞非曝露群であった．これらの結果は，前年度で得られた結果とはやや異なっていたが，いずれも，メスのみで影響が認められた点は一致していた．

厚生労働科学研究費補助金（化学物質リスク研究事業）

総括研究報告

Effects of Low-dose Perinatal Cadmium Exposure on Tissue Zinc and Copper Concentrations of Neonates and on Reproductive Development of Female Offspring in Mice　（低濃度の周生期カドミウム曝露がマウス新生仔組織の銅，亜鉛濃度ならびに性成熟に及ぼす影響）

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研究要旨：昨年度までの結果により，周生期における低濃度のカドミウム曝露がマウスにおいて発達毒性を有する可能性が強く示唆された．カドミウムが母体‐胎仔・新生仔系において毒性を発揮するメカニズムは不明であり，他の項でマイクロアレイなどを用いて検討を行っているが，良く知られているように，カドミウムが亜鉛・銅の代謝に影響を及ぼすことが毒性発現につながるという可能性がある．昨年度，この可能性については若干の検討をおこなっているが，本年度は検討時期を2点設け，より詳細に検討した．また，カドミウムがエストロゲン様作用を発現するという最近の報告も参考にして，性成熟への影響も検討した．

　これまで多くの検討は，飲料水にCdを添加する方法を採用したが，添加濃度は50ppmを超えるものが多く，飲水量や体重抑制などの指標から判断すると，これは明らかな毒性を生ずる量であって，発達毒性の検討には向いていない．本研究では，添加濃度をこれまでの検討で用いてきた10ppmに加えて，1ppmの2段階として，在胎期‐出生10日後まで母親を通じて曝露した．その結果，新生仔肝・腎において，高濃度の曝露で報告されているのとは逆に．Znの軽微な増加を認めた．一方で，肝におけるCu濃度の低下が認められた．これらの結果は，低濃度のCd曝露が微量元素の動態に影響を及ぼす可能性を示しているが，毒性との関連は明確ではない．性成熟については，Cd曝露が性成熟に影響する可能性を示唆するデータが得られたが，エストロゲン様作用を示した報告における性成熟への影響とは，影響の方向が異なっていた．

Introduction

In human pregnancy, maternal exposure to Cadmium (Cd) is associated with low birth weight (Kuhnert et al., 1988; Frery et al., 1993) and possibly with an increased incidence of spontaneous abortion (Shiverick and Salafia, 1999). Cd has also been shown to produce a variety of adverse reproductive outcomes in exposed animals. Cd administration to rodents impairs implantation (Balanski et al., 1982; Yu et al., 1985), decreases litter size (Balanski et

al., 1982; Samarawickrama and Webb, 1981), produces resorptions (Samarawickrama and Webb, 1981; Machemer and Lorke, 1981; Gale and Layton, 1980) and causes fetal or embryonic death (Ferm, 1971; Levin and Miller, 1980; Rohrer et al., 1979) . Fetal growth retardation (Hastings et al., 1987; Ahokas et al., 1980; Baranski et al., 1983; Baranski, 1987) and congenital malformations (Gale and Ferm, 1973; Chernoff, 1973; Barr, 1973) in the offspring of exposed rats have been the most widely reported adverse reproductive effects.

The passage of maternally administered Cd to offspring is different depending on the developmental stage. Cd administered to pregnant animals is possibly transferred to fetus via placenta, and to neonates through milk. At low to moderate doses during pregnancy, the placenta acts to restrict the entry of Cd into the fetus (Sonawane et al., 1975; Whelton et al., 1993). However, Cd can cross the placental barrier if the dose is sufficiently elevated (Sonawane et al., 1975). The transfer of Cd to milk is also restricted (Bhattacharyya, 1983; Pitrzak-Flis et al., 1978). Of the small amount cadmium passed from the dams to offspring during gestation and lactation, the major portion has been shown to be transferred during lactation in mice (Whelton et al., 1993). Moreover, Cd level in the brain increases after neonatal exposure to 50 ppm Cd (Gupta et al., 1993), which is different from the result of gestational exposure. However, the entry of Cd to neonatal brain has not been fully elucidated at lower dose of Cd during lactation.

The mechanism of Cd-mediated fetotoxicity is not fully understood. However, there are some data suggesting that toxic effects of Cd may be mediated by altered Zinc (Zn) and Copper (Cu) metabolisms (Baranski, 1986; Kuhnert et al., 1987; Sorell and Graziano, 1990; Suzuki et al., 1990). Adequate availability of both Zn and Cu is essential for normal growth and development. Insufficient Zn availability in fetal or early postnatal life is teratogenic (Hurley and Swenerton, 1966), retards growth (Sandstead et al., 1972; Beach et al., 1980) and alters cognitive function (Sandstead et al., 1972). Fetal or neonatal Cu deficiency is also teratogenic (Keen et al., 1982), reduced brain catecholamine levels (Morgan and O'Dell, 1977; Feller and O'Dell, 1980) and decreases myelination in the central nervous system (Dipaola et al., 1974; Zimmerman et al., 1976). Gestational exposure to oral Cd over 50 ppm in drinking water resulted in decreased Zn and Cu content in fetal liver (Baranski, 1987; Sorell and Graziano, 1990; Sowa and Steibert, 1985; Roelfzema et al., 1988, 1989), brain (Baranski, 1987; Sowa and Steibert, 1985), kidney and intestine (Sowa and Steibert, 1985), as well as in whole body (Pond and Walker, 1975; Petering et al., 1979) and in these tissues of neonates (Baranski, 1986) or adult offspring (Baranski, 1986; Roelfzema et al., 1989). However, both increased (Waalkes et al., 1982) and unaltered (Sowa et al., 1982) concentrations of Zn and Cu in the fetus have been reported following maternal Cd exposure. Moreover, little is known about the effects of perinatal Cd exposure on offspring at the level lower than those used in the previous studies.

Recently, estrogen-like activity of cadmium has been suggested by Garcia-morales et al(Garcia-Morales et al., 1994), who showed that Cd mimicked the effect of estrogens by decreasing the level of estrogen receptor mRNA and transcription of the ER gene. In addition, female offspring, exposed in ureto to Cd, experienced an earlier onset of puberty (indicated by an earlier vaginal opening) and an increase in the epithelial area and the number of terminal end buds in the mammary glands (Johnson et al., 2003). This showed that only two injections of Cd to dams at a dose of 0.5 or 5 μg/kg body weight can be enough to affect the reproductive organs of offspring. On the other hand, effects of Zn and/or Cu conditions on sexual maturation and reproductive development have not been reported until now.

Therefore, this study aimed to investigate the effects of Cd at a dose of 1 or 10 ppm in drinking water during pregnancy and early lactation period on the concentrations of Cd, Zn and Cu in the brain, kidney and liver of mouse neonates. The doses were chosen because very few have examined the effects of Cd in the range exceeding 10 ppm in drinking water. In addition, we measured these metals at two time points (at birth and after ten days interval) to describe the effects along the developmental stage. We also evaluated the effects of perinatal Cd exposure with these doses on vaginal opening and estrus cycle for the first time.

Materials and methods

Animals and treatments

Forty female C57Bl/6J Jcl mice of initial body weight 17-22 g were purchased from a commercial breeder (CLEA Japan, Inc.). After acclimatization for two weeks, female mice were mated with males (body weight 20-25 g) of the same strain (1:1). The onset of pregnancy was confirmed by the presence of plug in the vagina (defined as gestational day (GD) 0). Mating male mice were removed and 18 pregnant mice were housed individually in disposable cages (CLEA Japan, Inc.) on sawdust bedding and had free access to chow with reduced amounts of estrogen and phytoestrogen; 17ß-estradiol was less than 0.05 µg/kg, genistein was less than 0.5 mg/kg, and coumestrol was less than 1 mg/kg (NIH07-PLD; based on an open formula of National Institute of Health, CLEA Japan, Inc.). Rooms were kept at 23 ± 2 °C with a cycle of 12 h of light and 12 h darkness; the lights came on at 0700.

Pregnant mice were divided into three groups. The animals of the treatment groups received 1 (n=6) or 10 (n=6) ppm Cd as cadmium chloride in drinking water from GD 0 to postnatal day (PND) 10 (the day of birth was defined as PND 0), respectively. The control group (n=6) received only distilled water. From PND 10, animals of all groups were given distilled water to drink. Each cage was checked every morning for the presence of newborns and the day of parturition, the number of living pups, the number of stillborn pups (defined as dead pup when we newly found parturition), sex ratio and the body weights per litter were recorded. Pups were left with each dam until weaning at PND 21. At weaning, the littermates were separated and housed by sex. Female offspring were housed individually after vaginal opening. All pups were weighed every 10 days until PND70. We also checked the number of death during this period.

All experimental protocols were approved by the Animal Care Committee of the Graduate School of Medicine of the University of Tokyo.

Sexual maturation

After weaning, all female offspring (n=5, 12 and 5 for control, 1 ppm and 10 ppm group, respectively, and n=1-5/dam) were checked for vaginal opening every day. The day of and the body weight at vaginal opening were recorded.

Beginning with PND 50, vaginal smears were taken daily for fifteen days, and estrus cycle was monitored. The days from diestrus to metestrus was defined as the duration of one cycle. One cycle with more than six days was defined as longer cycle. If a set of smear from giver mouse did not show the order of diestrus-proestrus-estrus-metestrus, this mouse was judged to be without cyclicity. Thus, individual female offspring was classified into one of the three categories; without cyclicity, longer duration of one cycle and normal cyclicity (other than without cyclicity and longer duration of one cycle). Data with plural cycles per individual during the observation period were averaged within the individual for statistical analysis.

Metal measurements

One male and one female pups from each litter were sacrificed at PND 0 and 10 under diethyl ether. Brain, kidney and liver were removed and weighed. All dams were sacrificed at PND 21 likewise, then cerebrum, cerebellum, brainstem, kidney and liver were removed and weighed. Samples were ashed with 60% nitric acid and 60% perchloric acid (2:1) at 110 °C for 5 h. Ashed samples were diluted with distilled water. Tissue Cd, Zn and Cu concentration was determined with a Inductively Coupled Plasma Mass Spectrometer (Hewlett Packard - 4500 ICP-MS, USA). Calibration curves were made using ICP multi-element standard solution for MS (MERCK,

Germany). Three replicas were performed per sample. Relative standard deviation was less than 5%. As reference material, standard reference material 1577b bovine liver (National Bureau of Standards) was analyzed together with the samples. Results from the analysis of the reference materials were well within ± 10% of the certified values. Reagent blanks were processed with each set of samples. Detection limits for tissue were 2 ng/g for Cd, 35 ng/g for Zn and 8 ng/g for Cu. Some samples were under detection limit on Cd measurement, and the values for those samples were applied the half of the detection limit when statistical analysis was performed.

Statistical Analysis

Frequencies were analyzed with Pearson chi-square test. Values of averaged parameters were statistically evaluated with a one-way ANOVA followed by Dunnett test. In comparison of the metal concentrations of offspring between the two time points, Paired t-test was used. P values less than 0.05 were considered statistically significant.

Results

Reproductive performance of the dams

The weight gain in dams during pregnancy was not different among groups. Table 1 shows the data on the reproductive performance of the dams. All these parameters were not significantly different among groups. However, the number of death after birth tended to increase in the Cd-treated groups.

Growth and sexual maturation of offspring

There were no significant differences in the body weight grain between groups both in male and female offspring until PND 70.

Sexual maturation and function are shown in Table 2. Though there was no statistical difference, the vaginal opening tended to be later than the control group by one and two days in 1 and 10 ppm Cd group, respectively. Body weight at vaginal opening did not differ between groups. Two female offspring in 10 ppm Cd group did not show cyclicity, resulting in a significant difference in the frequency of offspring without cyclicity (p=0.024 by Pearson chi-square test). We excluded these two offspring from analysis on the duration of one cycle. No significant difference was found in the duration of one cycle between groups.

Metal concentrations in dams

Tissue concentrations of Cd in dams are shown in Fig. 1. Cd concentration in the kidney [F(2, 12)=9.14, p<0.0001] and liver [F(2,12)=33.54, p<0.0001] was higher in 10 ppm Cd group compared with the other groups (Fig. 1A), while no between-group difference was found in the brain regions examined (cerebrum, cerebellum and brainstem) (Fig. 1B). Neither Zn nor Cu concentrations in the examined tissues differed among the groups.

Metal concentrations in offspring

Fig. 2 shows the metal concentrations of the neonatal tissues on PND 0. Between-group difference in Cd concentration was significant in the brain [F(2, 27)=5.62, p=0.009] and marginally significant in the liver (Fig. 2A). Zn concentration in the kidney [F(2, 30)=3.49, p=0.043] and the liver [F(2,29)=4.48, p=0.019] was different among groups, while it was not in the brain (Fig. 2B). Cu concentrations in the tissues of offspring did not differ among groups (Fig. 2C).

Fig. 3 shows the metal concentrations of offspring at PND 10. Difference in Cd concentration was found in the kidney [F(2, 17)=4.05, p=0.036] and liver [F(2,17)=3.78, p=0.049], and the difference was marginal in the brain (Fig.

3A). No between-group difference in Zn concentrations were found in the tissues examined (Fig. 3B). Cu concentration in the kidney [$F_{(2,17)}=4.02, p=0.037$] and the liver [$F_{(2, 17)}=3.80, p=0.043$] showed the difference among groups (Fig. 3C). In the liver, Cu concentration was significantly lower in the offspring of 10 ppm group than control. No such difference among the groups was found in the brain. Neither sex differences in the concentrations of these metals were recognized.

Changes of the metal concentrations were recognized in the neonatal tissues between PND0 and PND10. In the brain, Cd and Cu concentrations were significantly decreased from PND0 to PND10 in control group ($p=0.032$ and $p=0.023$, respectively) but not in the Cd-treated groups, while Zn concentrations were decreased in all three groups ($p=0.014, p=0.002$ and $p=0.042$ for control, Cd 1 ppm and 10 ppm group, respectively). In the kidney, only the Cd concentration in the control group was decreased ($p=0.035$). In the liver, Zn concentration was significantly decreased only in the control group ($p=0.005$), whereas Cu concentrations were decreased in all the groups ($p=0.005, p=0.014$ and $p=0.022$ for control, Cd 1 ppm and 10 ppm group, respectively).

Discussion

In the present study, perinatal exposure to low-dose Cd increased Cd concentrations in the brain of offspring brain at PND0, as well as the kidney and the liver at PND10. The Cd exposure also increased hepatic and renal Zn concentrations in the offspring at PND0, a novel finding suggesting the effect of Cd may not be solely leading to the deficiency of this trace elements as shown with many reports employing higher doses of Cd. Cu concentration in Cd exposed offspring was higher in the kidney and lower in the liver. Furthermore, we suggest the possibility that the onset of puberty can be delayed in female offspring by the Cd exposure, and this is the first report to show the effect of perinatal Cd exposure on estrus cycle of female offspring.

Cd concentration in the brain of Cd-exposed offspring was significantly higher than that of control at PND0, and had a higher tendency at PND10. The latter agreed with several previous studies showing the increased levels of Cd in the brain of offspring after neonatal exposure (Gupta et al., 1993; Choudhuri et al., 1996; Valois and Webster, 1987; Wong and Klaassen, 1980, 1982). However, studies employing a wide range of dose (up to 180 ppm in drinking water) and varying exposure paradigms have not found elevated levels of Cd in the fetal brain (Baranski, 1987; Sowa and Steibert, 1985; Murthy et al., 1986). This is explained by the placental barrier to restrict the entry of Cd to the fetus (Sonawane et al., 1975). Cd enters the brain most freely right from birth with entry decreasing until access is basically restricted around PND21 (Hastings and Miller, 1998). This can be accounted for, in some part, by the formation of a well-developed blood-brain barrier with age. Lucis *et al.* (Lucis et al., 1972) reported that Cd penetrates the blood barrier with more ease in fetal rats than in adults. Considering together, our result suggests that although the transfer of Cd from mother to offspring via placenta is restricted, Cd can still cross the placenta and accumulate in the brain at birth even at low level. Even though the development of blood-brain barrier with age is a factor for the increased exclusion of Cd (Hastings and Miller, 1998), brain Cd level at PND10 was still higher due to an immature blood-brain barrier.

Cd concentrations in the kidney and the liver of offspring did not differ among groups at PND0, which is consistent with the previous study using 5 ppm Cd in drinking water during gestation (Sorell and Graziano, 1990). However, when dams were given 50-180 ppm Cd in drinking water during gestation, fetal liver at GD20 showed the higher concentration of Cd (Baranski, 1987; Sorell and Graziano, 1990 ; Roelfzema et al., 1989). On the other hand, Cd concentrations in these tissues were higher in Cd-exposed groups at PND10. Although Cd transfer via milk has reported to be low in mice and rats (Lucis et al., 1972), the present study showed the importance of lactational transfer of Cd at such low level; this is consistent with the results of preceding studies (Whelton et al., 1993; Petersson et al.,

2000).

Zn concentrations in the kidney and in the liver were higher in 10 ppm Cd group than control at PND0. This result was inconsistent with the previous studies dosing over 50 ppm Cd, which reported decreased Zn and Cu content in fetal kidney (Sowa and Steibert, 1985) and liver (Baranski, 1987; Sorell and Graziano, 1990; Sowa and Steibert, 1985; Roelfzema et al., 1988). In rats, the maternal Cd exposure produced a decrease in fetal hepatic metallothionein (MT) levels with decrease in Zn level/concentration (Waalkes and Bell, 1980; Sasser et al., 1985). On the other hand, hepatic Zn increases in the rabbit fetus following maternal Cd exposure, where simultaneous increase in MT was observed (Waalkes et al., 1982). Although we have not determined whether the increase in Zn concentration in neonatal organs were related with the induction of MT, this result for the first time suggested that the response of mice were similar to that of rabbits. Although the fundamental cause of such species difference is not known, this should be clarified with further researches. Furthermore, Zn concentration in the liver of control offspring decreased from PND0 to 10. Previous studies also found the decrease in hepatic Zn concentration with age after birth in rodents (Reis et al., 1991). On the other hand, Zn concentrations in the liver of exposed offspring did not change during this period, suggesting that maternal low-dose Cd exposure promotes Zn retention in the liver of offspring.

Cd accumulation did not affect the Zn and Cu concentrations in the kidney and liver of the dams. This is inconsistent with some of the previous studies using higher Cd doses (i.e., 50-200 ppm Cd in drinking water), in which increases in hepatic and/or renal Zn (Sorell and Graziano, 1990; Pond and Walker, 1975; Chmielnicka and Sowa, 1996) or Cu (Chmielnicka and Sowa, 1996) concentration have been noted in pregnant rats. On the other hand, as discussed above, the increased Zn concentrations in the kidney and the liver of offspring were observed in the absence of significant Cd accumulation in these tissues at PND0. Christley and Webster (Christley and Webster, 1983) demonstrated that Cd does not necessarily have to be present in tissue at detectable levels to produce toxicity. Furthermore, studies involving Cd exposure during gestation usually demonstrated alterations in essential trace metals even though there was no increase in Cd (Hastings and Miller, 1998). Therefore, it is suggested that undetectable difference of Cd level between the Cd-exposed and control groups might affect Zn metabolism in the kidney and the liver of the neonates, and/or that alteration of Zn metabolism by Cd are more sensitive in fetuses/neonates than adults.

Finally, we found a tendency that the day of vaginal opening was later in female offspring born to Cd-treated dams. Furthermore, two female offspring of the 10 ppm Cd group did not show estrus cyclicity at all. Salvatori *et al.* (Salvatori et al., 2004) also showed a delay of vaginal opening in rats prenataly exposed to Cd. On the other hand, an earlier onset of vaginal opening was reported in rats with much lower dose (Johnson et al., 2003). This suggests that Cd has estrogen-like activity, because *in utero* exposure to estrogens or estrogen-like substances causes early onset of puberty (Hilakivi-Clarke et al., 1997; Rothschild et al., 1988). However, female rats prenataly exposed to genistein had delayed puberty onset, and those exposed to diethylstillbestrol had atypical vaginal cycles (Levy et al., 1995). They suggested that prenatal exposure to estrogen-like substances including genistein might cause a delay in puberty onset by an inhibitory or androgenizing effect on the hypothalamo-hypophysial axis, which controls estrogen production leading to initiate vaginal opening. Moreover, disrupted estrus cycle and anovulation were shown to be characteristic responses to estrogen exposure in rodents during critical periods of neuroendocrine differentiation (earlier than 5 to 10 days of age) (Gellert, 1978). Therefore, our data supported the notion that perinatal low-dose Cd exposure would perturb the reproductive-endocrine function. In this study, we could not find the clear relations between vaginal opening/estrus cycle and Zn/Cu concentrations.

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Table 1.Effect of Cd exposure to the dams during pregnancy and early lactation period on reproductive performance

	Control	1 ppm Cd	10 ppm Cd
Period of gestation (days)	19.7 ± 0.5	18.8 ± 0.2	21.1 ± 1.1
Litter size	5.8 ± 1.1	7.7 ± 0.5	5.8 ± 0.7
Sex ratio (males to females)	1.29 ± 0.25	1.02 ± 0.39	1.00 ± 0.52
Birth weight (g)	1.303 ± 0.016	1.298 ± 0.009	1.300 ± 0.018
Livebirth (n)	35	46	34
Stillbirth (n)	3	2	1
Death after birth (n)	6	11	13

Values are expressed as mean ± SE for period of gestation, litter size, sex ratio and birth weight.
n=6 for control, n=6 for 1 ppm Cd group and n=6 for 10 ppm Cd group.

Table 2.Effect of Cd exposure to the dams during pregnancy and early lactation period on sexual maturation and function of female offspring.

	Control	1 ppm Cd	10 ppm Cd
Day of vaginal opening (PND)	30.2 ± 1.2	31.4 ± 0.7	32.4 ± 1.2
Body weight at vaginal opening (g)	15.00 ± 0.10	15.41 ± 0.28	15.52 ±0.49
Duration of one cycle (days)	5.23 ± 0.32	5.72 ± 0.50	5.29 ± 0.49
Estrous cycle			
No. of offspring with normal cyclicity (	3	10	2
No. of offspring with longer cycle (n)	2	2	1
No. of offspring without cyclicity (n)	0	0	2*

Values are expressed as mean ± SE for day of vaginal opening, body weight at vaginal opening and duration of one cycle.
n=5 for control, n=12 for 1 ppm Cd group and n=5 for 10 ppm Cd group.
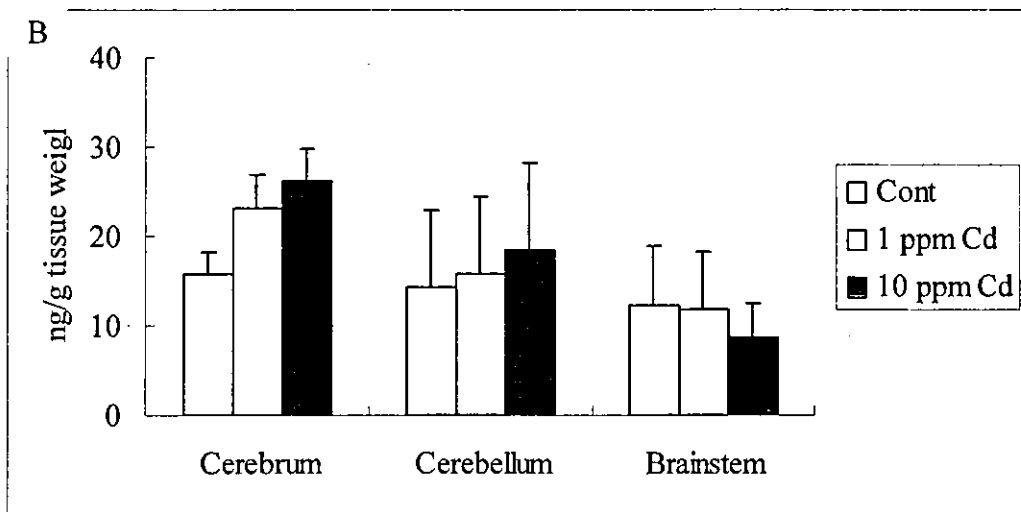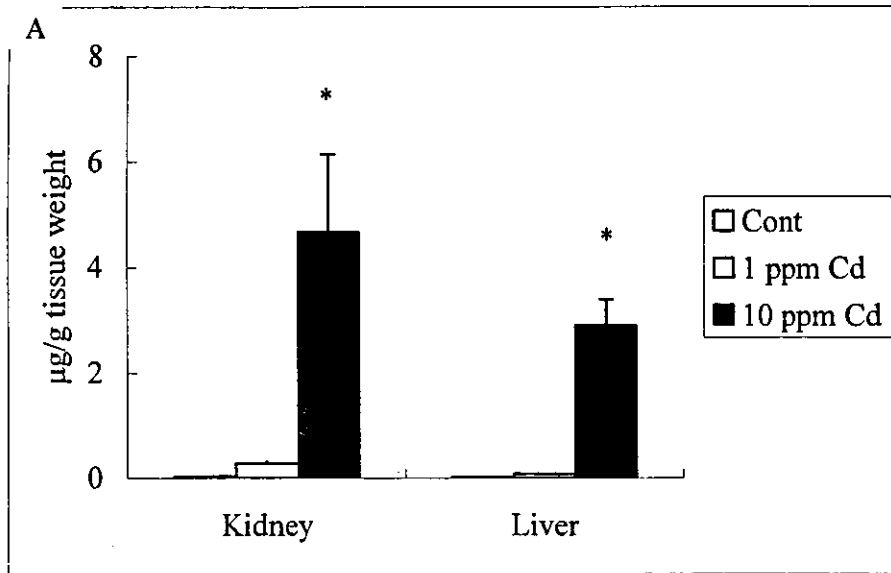*; $p < 0.05$ compared with control

Fig 1. Effect of Cd exposure to the dams during pregnancy and early lactaion perios on Cd concentration in the kidney, the liver (A) and the brain (B) of dams.

Data are expressed as mean ± SE.

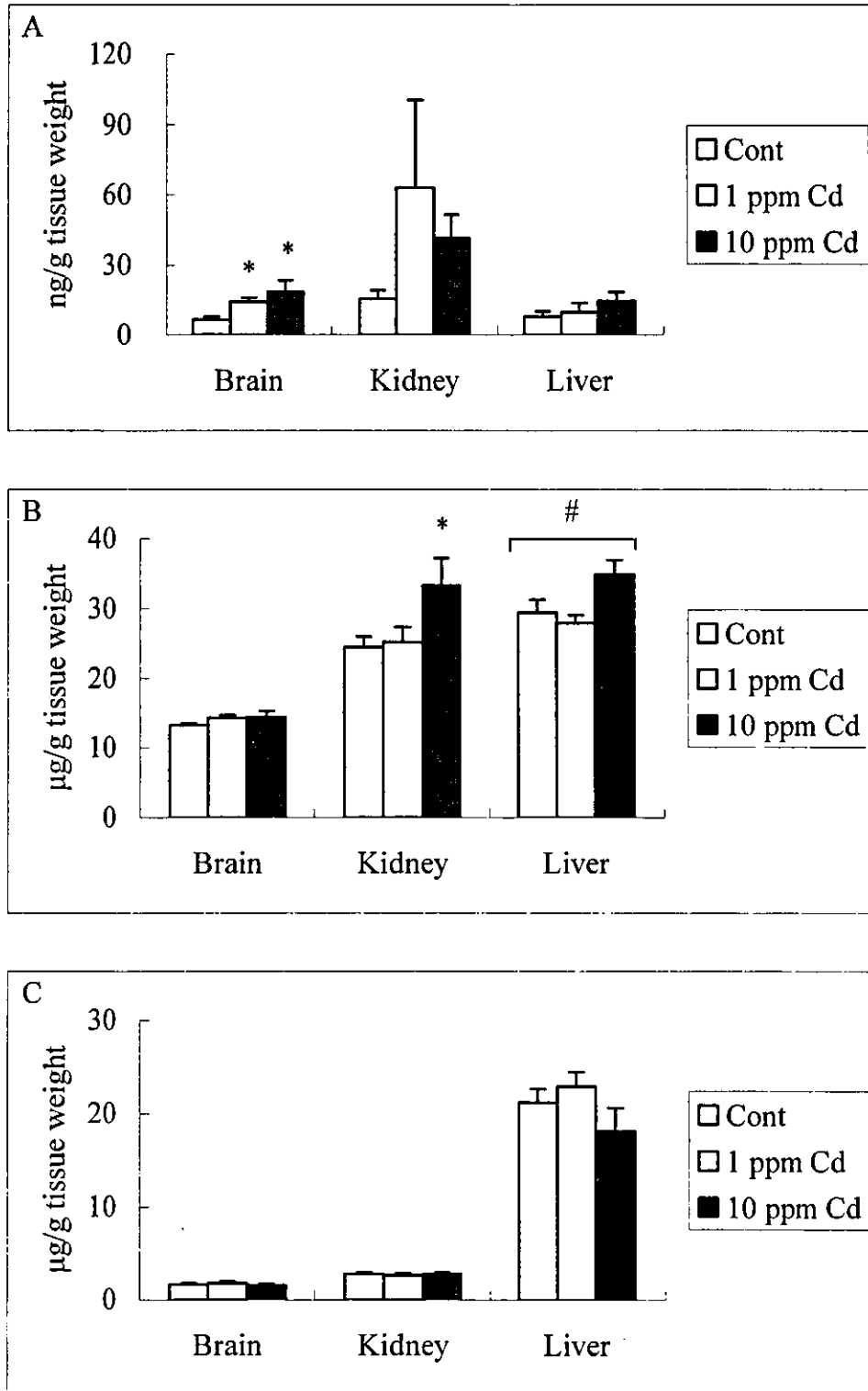*; p<0.0001 compared with control.

Fig 2. Effect of Cd exposure to the dams during pregnancy and early lactation period on
Cd (A), Zn (B) and Cu (C) concentrations in tissues of offspring at PND0.

Data are expressed as mean ± SE.

#; $p < 0.05$ different from each other by one-way ANOVA

*; $p < 0.05$ compared with control