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Original Article

Sexually immature male ERE-*Luc* reporter mice to assess low dose estrogen-like effects of $CdCl_2$ *versus* dietary Cd

Balaji Ramachandran[1], Nicoletta Rizzi[1], Adriana Maggi[1,2]

[1]*Center of Excellence on Neurodegenerative Diseases and Department of Pharmacological Sciences, University of Milan, Via Balzaretti, 9 I-20123 Milan, Italy;* [2]*Animal Models Platform Fondazione Filarete, Milan, Italy*

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Abstract: $CdCl_2$ salt is widely used in exposure oriented studies, while the biological exposure of Cadmium (Cd) occurs mostly through diet. Hence, we designed a *in vivo* imaging methodology with sexually immature male ERE-*Luc* reporter mice to test the estrogen-like (EL) effects of Cd as a natural component in wheat and flax bread based diets (containing 17.57 and 49.22 ug/kg Cd concentrations respectively) and $CdCl_2$ *per-oral* dose of 1 ug/kg/bw/day. Total exposure of ingested and % bioaccumulation of Cd in selected organs were estimated as 547 ng (4.4%), 776 ng (0.3%) and 2131.8 ng (0.1%) corresponding to $CdCl_2$, wheat and flax bread based diet treatments respectively. Cd from $CdCl_2$ bioaccumulated more readily, despite the exposure of Cd is higher with bread based diets. Longitudinal *in vivo* imaging did not reveal significant changes in luciferase activity. White adipose tissue (WAT) and prostate were identified as novel target organs of Cd. Indeed, the rest of the observed EL effects, endogenous target gene expression and necropsy findings are not consistent to any particular organ or treatment. This implies that, the observed EL effects due to low doses of Cd (either from $CdCl_2$ or dietary form) occur only as subtle changes at the molecular level, but inadequate to cause significant changes at the anatomo-pathological level during the 21 day exposure period. The study demonstrates the sensitivity of the methodology to assess EL effects of food embedded Cd and underlines the limitations of directly extrapolating the results of suspected chemicals in their pure form to dietary exposure scenarios.

Keywords: Reporter mice, *in vivo* imaging, estrogenicity, $CdCl_2$, dietary Cd, bioavailability, endocrine disruptors

Introduction

Emerging evidences show that, Cd is clearly a potent non-steroidal estrogen *in vivo* [1-6] and *in vitro* [7-9]. A large body of evidence points to a major endocrine disruptive action of this heavy metal due to its ability to bind with high affinity to estrogen receptor alpha (ER-α), independently of estrogen binding and to activate this receptor [1, 7, 8, 10]. In most *in vivo* studies, Cd effects were analysed by administering $CdCl_2$ [1-4] and this type of treatment does not really mimic the effects of dietary exposure to this metal.

Food accounts for approximately 90% of human exposure to Cd in the non-smoking general population and the major foodstuffs that contribute to this exposure are cereal products, vegetables, nuts and starchy roots (http://www.chem.unep.ch/Pb_and_Cd/SR/Draft_final_reviews/Cd_Review/Final_UNEP_Cadmium_review_Nov_2008.pdf), [11]. However, Cd is present in virtually all foods at variable concentrations, depending on the type of commodity and the extent of environmental contamination [12, 13]. Bread, accounting for 36% of the weekly intake, has been identified as the biggest single source of Cd in diet in Sweden [14]. Cd absorption after dietary exposure in adults is relatively low (5-10%), but may be higher in infants [15]. Once absorbed, Cd is retained in kidney and liver, and has a very long biological half life, ranging from 10 to 30 years see [5] for details on toxico-kinetics.

Secondly, gender differences exist in the bioaccumulation and toxicity of absorbed Cd [16, 17]. Although females are reported to retain higher liver and kidney concentrations than males

[13], males are less vulnerable to Cd related bone effects compared to females [18], but are much prone to Cd related reproductive damage [19, 20] affecting semen quality, testis and epididymis [21]. In the specific case of immature males, even though the new born epididymis show resistance to Cd, the effects become more evident in adult hood [22]. Cd accumulation is toxic to the gonads of male reproductive apparatus from embryo till adult hood [23, 24]. However, the severity of the testicular damage increases with age [25]. Cd is also shown to be neurotoxic to the developing brain [26, 27]. Oral and chronic Cd intake was shown to induce neoplastic and proliferative lesions in adrenal, kidney, prostate, pituitary and testis of experimental animals [28, 29]. Cd was reported to produce neoplasm in the prostate of rats or changes in sperm counts in a dose dependent fashion at a low dose exposure that is far below the threshold for significant testicular toxicity [30, 31]. Low dose effects occur at a range of human exposure and at a range below the no adverse effect level (NOAEL) where, various irreversible effects are reported [32]. The effects are dependent on the nature of compound studied [33, 34] age and sex [35] and are either linear, threshold appearing or non-monotonic [36]. A health based guidance value for Cd of 7 μg/kg/bw/week or 1 μg/kg/bw/day was established as Provisional Tolerable Weekly Intake (PTWI) by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO, 2005 ftp://ftp.fao.org/es/esn/jecfa/jecfa64_summary.pdf). The tolerable weekly intake (TWI) for Cd was established by European Food Safety Authority (EFSA) [11] to be of 2.5 μg/kg/bw. EFSA estimated the median average dietary exposure across european countries is between 2.3-3.0 μg/kg/bw/week. In 2010, JECFA (http://www.who.int/entity/foodsafety/publications/chem/summary73.pdf) reviewed its previous evaluation on Cd and established a provisional tolerable monthly intake (PTMI) of 25 μg/kg/bw which corresponds to a daily intake of 0.82 μg/kg/bw. However, daily human intake of Cd is between 0.1 ug/kg/bw (low exposure) and 10 ug/kg/bw (high exposure) [37]. Average daily dietary Cd intake in unpolluted european areas vary from 0.1 to 0.45 μg/kg/bw, but in polluted areas the total intake may be higher than the tolerable daily cadmium intake and reach several hundred ug/day [38].

Traditional studies of low doses of contaminants can be expected to miss such responses at physiological levels [39]. Because at low doses, any detrimental changes can be overcome by homeostatic regulation until exceeding threshold, due to continuous bioaccumulation of the toxicant. Therefore, a methodology to screen even subtle effects during long term exposure of low doses of contaminants in its inherent form, at physiological level is indispensable and is still lacking.

ERE-*Luc* reporter mouse was generated for the rapid, quantitative and systemic analysis of the ER activity in living mice in the presence or absence of exogenous estrogenic stimuli [40, 41]. Using sexually immature male ERE-*Luc* reporter mice [40] we generated a non-invasive bioluminescent *in vivo* imaging methodology to measure the changes in estrogenic activity in response to treatment for a period of 21 days. We investigated the EL effects of two cereal based diets which are known to concentrate environmental Cd and compared its effects with $CdCl_2$.

Materials and methods

Chemicals

$CdCl_2$ was purchased from Sigma-Aldrich (Pomezia, Italy), ketamine (Imalgene 500) from Merial (Toulouse France), xilazine (Rompun) from Bayer (Shawnee Mission, Kansas, USA), and D-luciferin (Beetle luciferin potassium salt) from Promega (Milan, Italy).

Experimental animals and rodent diets

The study was carried out using heterozygous 21 days old immature male ERE-*Luc* mice [40] in the C57BL/6 genetic background. Animal colonies were housed according to the Guidelines for Care and Use of Experimental Animals. All animal studies were approved by the Italian Ministry of Research (DM1 24/2003-A) and Milan University after approval by the expert committee at the Department of Pharmacological Sciences, University of Milan. The animal room was maintained within a temperature range of 22-25°C and relative humidity of 50%±10%. There was a cycle of 12 hours light/dark (lights on at 07:00 AM). The two types of bread based diets selected for the study were generated using white wheat toast

Table 1. Effect of 21 day treatment with $CdCl_2$ or bread based diets on animal body weight

Treatment	Control	$CdCl_2$	wheat bread	flax bread
Total water consumption (% of controls)	100	111	106	96
Total food consumption (% of controls)	100	87	93	87
Body weight (gr)[α]	20±0.3	17±0.2**	18±0.5	21±0.3

[α]Data represent the mean ± SEM (standard error mean) (n=5); **p<0.01 control *versus* treatment and time, by two-way ANOVA plus Bonferroni *post hoc*.

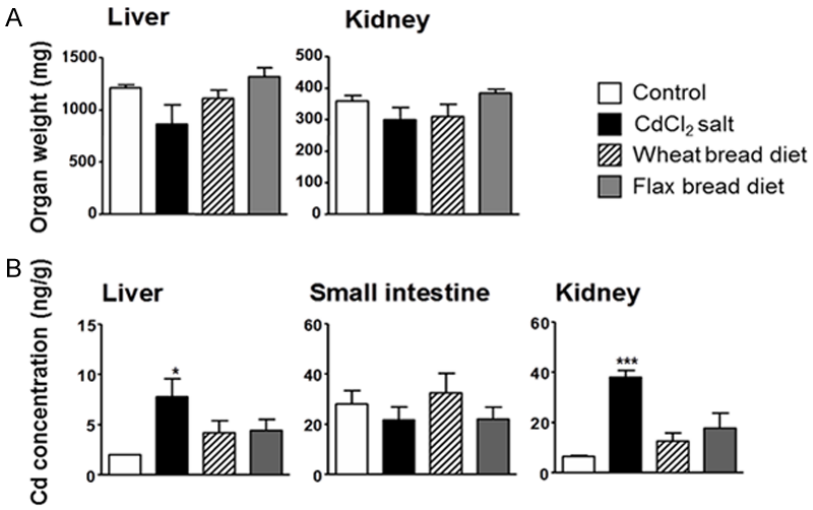

Figure 1. Cd accumulation in liver, small intestine and kidney of male treated for 21 days with $CdCl_2$ or two bread based diets. A: Fresh liver and kidney weight were measured after dissection. B: Tissue cadmium content was measured in liver, small intestine and kidney and data was expressed as ng/g of tissue. Data represents mean ± SEM (n=5) and asterisk indicates a significant difference from control, as assessed by one-way ANOVA plus *Bonferroni post hoc* test (*p<0.05), (***p<0.001).

bread and flaxseed supplemented white wheat toast bread containing 17.57 ug/kg and 49.22 ug/kg, of Cd respectively. The breads were incorporated into purified, balanced rodent diets. AIN93 based control diet contained 9.22 ug/kg of the heavy metal. Experimental rodent diets were prepared as mentioned in the results section of [5].

Study design

To lower the background exposure to dietary estrogens, the lactating female mice were switched to purified AIN93G diet one week after the delivery of the male pups. The pups are weaned at the age of 21 days and used for the experiment. Immature male mice were maintained in the same diet throughout the study (up to 42 day of age). The baseline luciferase activity before the start of the 21 day experimental study period was measured in all animals by *in vivo* imaging and the mice were matched for age, body weight and allocated into different experimental groups so that the average background luciferase activity of the chest area at the start of the experiment were comparable in all groups. 5 animals were used per treatment group and all the animals had free access to food and tap water for the entire duration of the study. Feed, water intake and body weight gain/loss were recorded once a week throughout the experiment. Since animals were maintained in cages of five mice each, we measured the entire amount of food and water consumed daily by the group and normalized to single animal.

The four different experimental groups were: (1) control group (purified AIN93G diet); (2) $CdCl_2$ group (1 ug/kg/bw/day *per-oral* dose); (3) wheat bread diet (low Cd content 17.57 ug/kg); and (4) flax bread diet (high Cd content 49.22 ug/kg). In the $CdCl_2$ group, $CdCl_2$ dissolved in saline, was administered as a single 100 µl bolus every 24 hours. The mice were maintained in the experimental groups for 21 days.

In vivo imaging sessions were carried out on days 0, 1, 7, 14 and 21 at 2:00 PM, before the daily $CdCl_2$ administration, as described before [42]. For quantification, photon emission was counted in the regions of interest and the signals obtained were integrated from each anatomical area as previously described [43, 44]. Photon emission is defined as the number of

Table 2. Total Cd ingested and % bioaccumulation in selected tissues after 21 day exposure $CdCl_2$ or bread based diets

Treatment	Oral intake ng	TOT Cd[a] in tissues[b], ng (% intake)	Cd in intestine[b], ng (% intake)	Cd in liver[b], ng (% intake)	Cd in kidneys[b], ng (% intake)
Control	363	-	-	-	-
Wheat bread	776	2.3 (0.3)	1.5 (0.1)	0.5 (0.06)	0.3 (0.04)
Flax bread	2132	2.5 (0.1)	-[$]	1.0 (0.04)	1.5 (0.07)
$CdCl_2$	547 (233 ng from the $CdCl_2$ and 314 ng from the AIN93G diet)	10.3 (4.4)[c]	-[$]	4 (1.71)[c]	6.3(2.71)[c]

[a]The average background concentration (i.e. average Cd content in the control group, 2 ng in the liver, 6 ng in the kidney, and 28 ng in the intestine) has been subtracted from the values; [b]Intestine, liver and kidneys; [c]% intake value is based on the intake of $CdCl_2$; [$]Values less than control background.

counts per second per square centimeter (Cts/Cm^2s). Quantifications were done using WinLight 32 imaging software (Berthold Technologies). Normalization was performed using an external source of photons enabling to measure the instrumental efficiency of photon counting (Glowell, Luxbiotech, Edinburgh, UK).

On the last day of the experiment, the animals were sacrificed and selected organs were excised and placed in a light-tight chamber for the ex-*vivo* measurement of tissue specific signals. Gray-scale images were first taken with dimmed light, and then photon emission was registered for 15 minutes. Merging of the pictures enabled to visualize the tissue specific localization of the photon emission signal in individual organs (luciferase signal was transformed in pseudo-colors: white-high > yellow > red > green > blue-low). Quantification was done as described above.

Dissected organs were equally divided for the quantitative analysis of the content of Cd, luciferase and specific mRNAs. All tissues were handled with acid washed material to avoid Cd contamination and the small intestine was washed with 0.9% NaCl containing 5 mM EDTA (pH 7) to remove any undigested food. Cd content measurement and gene expression analysis were carried out as described in the methods section of [5]. Quantitative enzymatic luciferase assay was carried out as previously described [44].

Anatomo-pathological analysis of experimental animals

Immature male mice at the end of the 21 day study (42 days of age) were euthanized by cervical dislocation and immediately underwent necropsy. The following organs/tissues have been sampled and fixed in 10% formalin: lungs, thymus, liver, spleen, hearth, kidney, testicles, epididymis, coagulating glands and seminal vesicles, urinary bladder and prostate. Histological samples were paraffin embedded, sectioned and stained with hematoxylin and eosin.

Necroscopy did not reveal any major change in the different experimental groups. None of the tissues examined showed lesions or morphological differences compared to mock treated controls.

Statistics

The statistical analysis was calculated as described [5].

Results

Food and water intake, body and organ weight measurements

We evaluated the effect of $CdCl_2$ and two different bread based diet treatments, upon food and water consumption every once a week in the immature male ERE-*Luc* mice during the 21 day study period (**Table 1**); Different treatments did not change the total food and water consumption. Interestingly, at the end of the 21 day study, mice treated with $CdCl_2$ showed a significant decrease in body weight. This effect was not observed in the animals fed with bread based diets (**Table 1**). We also measured the weight of liver and kidney and no change in organ weight was observed (**Figure 1A**). But we emphasize that a measure of organ weight is not a direct indication of toxicity associated with the treatment.

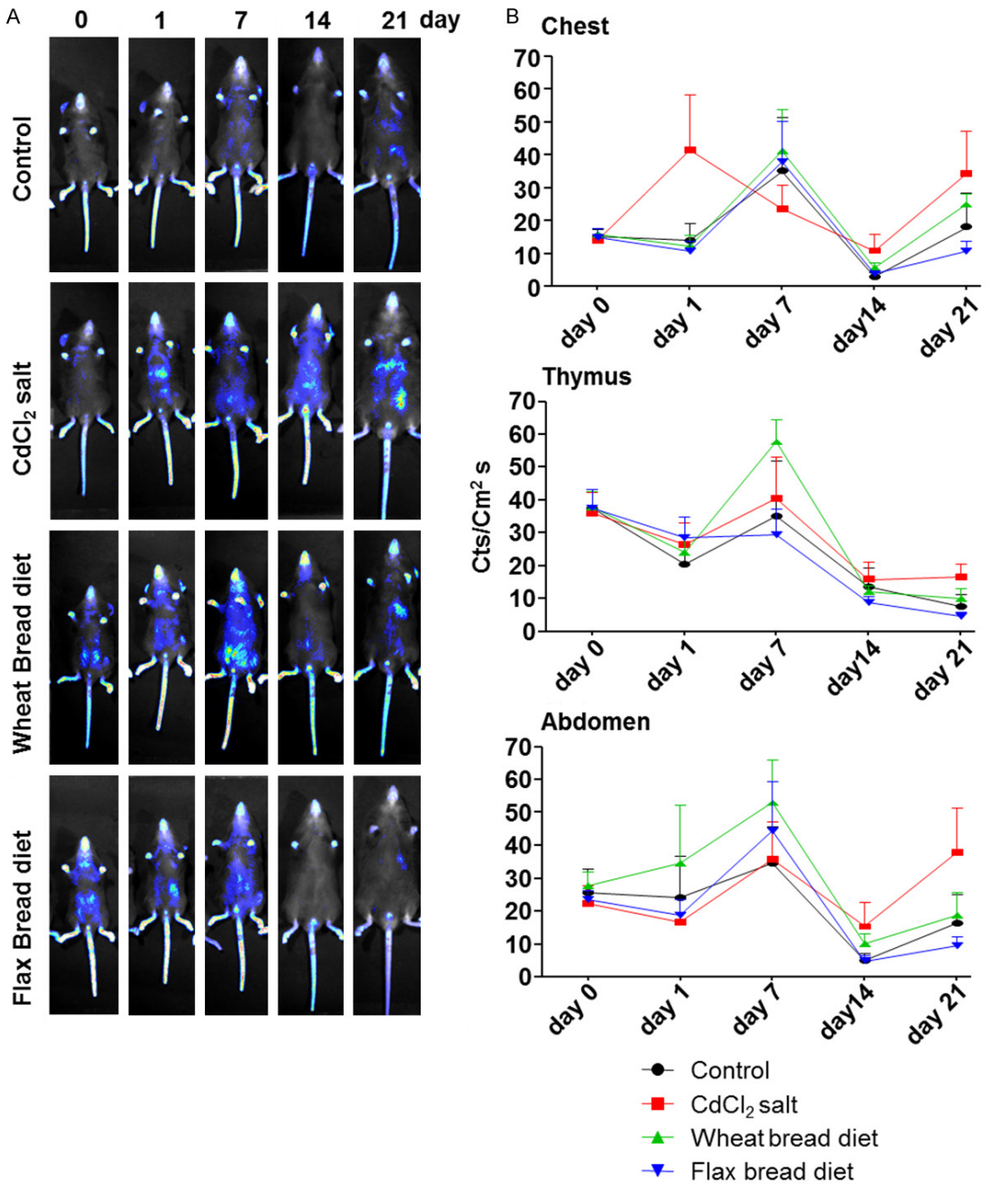

Figure 2. Bioluminescence *in vivo* imaging of male ERE-*Luc* mice treated with $CdCl_2$ and two bread based diets for a period of 21 days. A: Representative images of ERE-*Luc* mice at 0, 1, 7, 14 and 21 days of treatment. B: Quantitative analysis of photon emission from chest, thymus and abdomen of control and treated mice. Data represent the average of determinations made in groups of 5 animals each.

Cd content in liver, kidneys and intestine

Cd content was measured in liver, kidney and intestine, at the end of the 21 day treatment and in agreement with the trend observed in the previous report [45], it was found that Cd content was increased significantly in liver (+275%) and kidney (+381%) of the animals treated with $CdCl_2$, but not in the animals consuming the bread diets (**Figure 1B**). Accumulated Cd content from the intestines of $CdCl_2$ and flax bread diet treatment groups

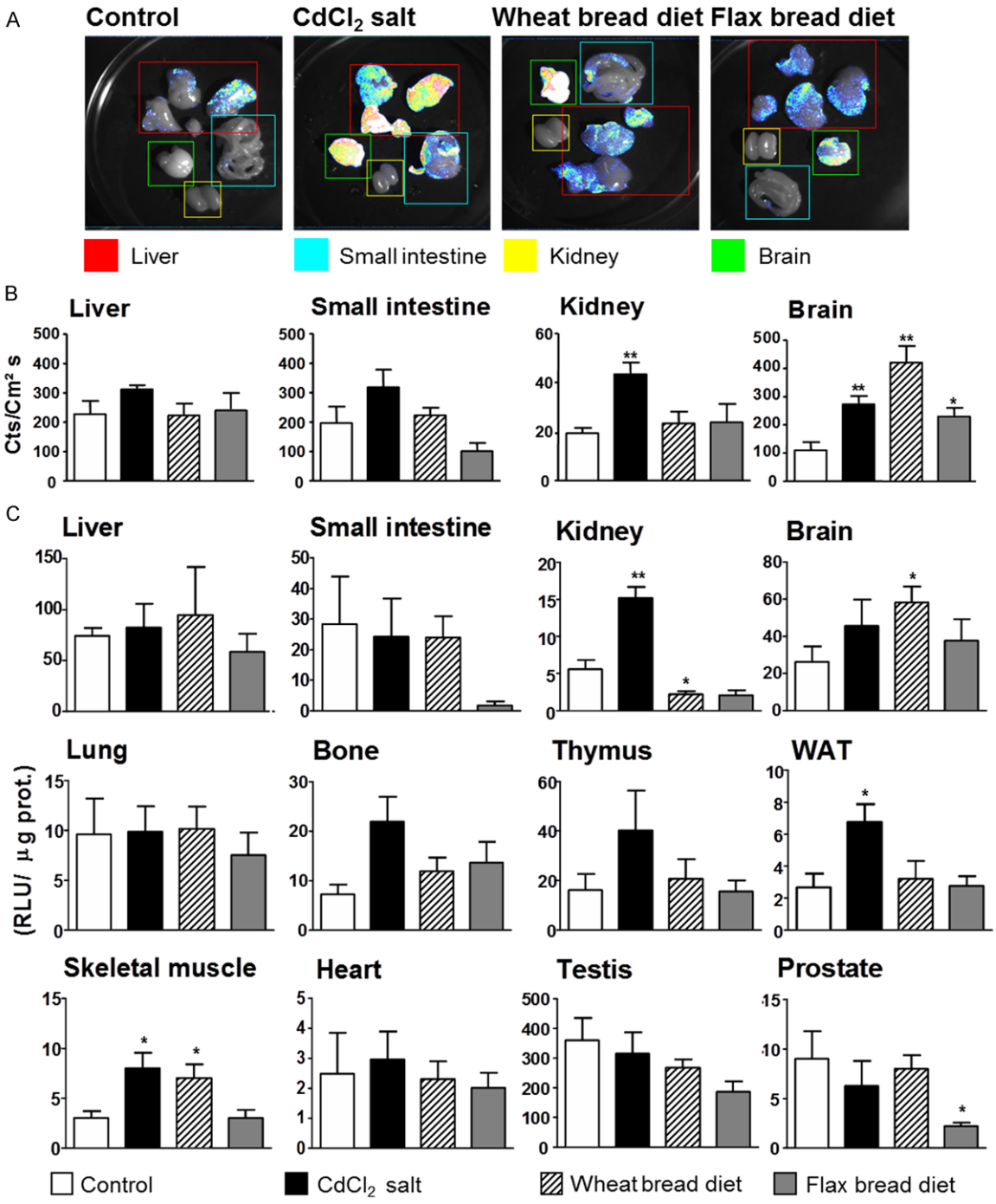

Figure 3. $CdCl_2$ or two bread based diets showing tissue specific pattern of ER activation in 21 day old male ERE-*Luc* mice. (A) Representative images of photon emission exposure in freshly excised liver, small intestine, kidney and brain as measured by CCD camera at the end of the 21 day long treatment. (B) Tissues were dissected from euthanized mice at the end of the treatment and ex *vivo* imaging analysis were measured in isolated tissues to identify the tissue specific localization of the luciferase signal. The emitted photons are counted and expressed as Cts/Cm^2/s. (C) Luciferase content of the dissected tissues were measured by enzymatic assay in tissue homogenates. Data in (A) and (B) were obtained from the same experiment; data represents mean ± SEM (n=5) and asterisk indicates a significant difference from control, as assessed by one-way ANOVA plus *Bonferroni post hoc* test (*p<0.05), (**p<0.01).

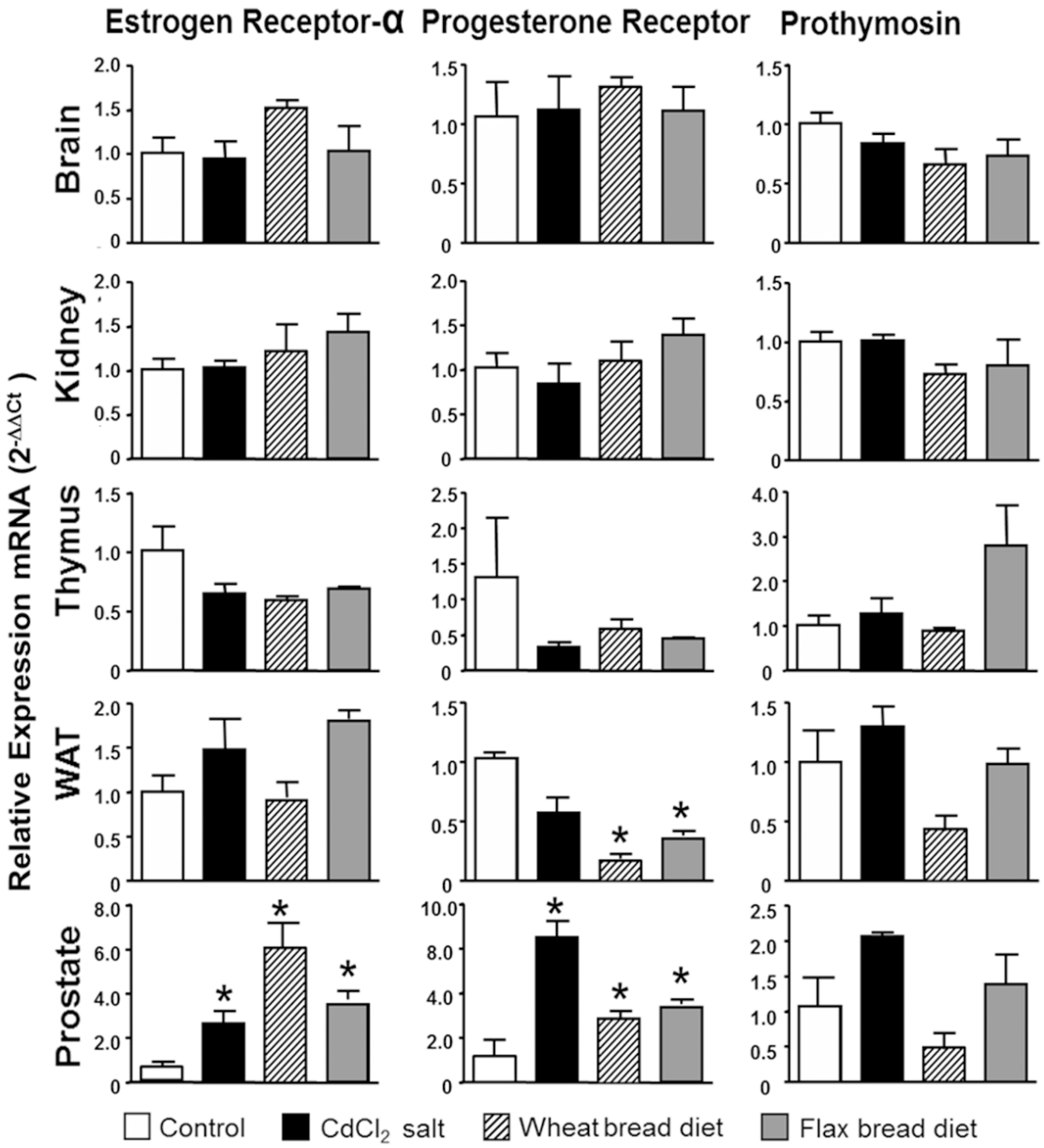

Figure 4. Effect of $CdCl_2$ or two bread based diets on expression pattern of ER-α and target genes in 21 day old male ERE-*Luc* mice. Data show relative levels of ER-α, PR and PTMA mRNAs in brain, kidney, thymus, WAT and prostate. The expression is shown relative to the ribosomal 18S RNA. data represents mean ± SEM (n=5) and asterisk indicates a significant difference from control, as assessed by one-way ANOVA plus *Bonferroni post hoc* test (*p<0.05).

were less than the background exposure. We estimated the total oral Cd intake from different treatments based on the recorded body weights and food consumption data. Throughout the 21 day study period, the control group mice ingested a total of 363 ng Cd, while wheat and flax bread diets treatment ingested 776 and 2132 ng of Cd respectively. The group treated with $CdCl_2$ ingested a total of 547 ng Cd (comprising of 233 ng Cd from $CdCl_2$ and 314 ng Cd from the ingested control diet) (**Table 2**). The amount of Cd retained in different organs was then calculated. Since the animals are young, we found very little background Cd concentration in the controls (average Cd content in control group, 2 ng in the liver, 6 ng in the kidney, and 28 ng in the intestine), which we subtracted from the corresponding treatments. Although, we fed animals with bread diets containing different concentrations of inherent Cd,

the amount of Cd accumulated in the tissues is negligible irrespective of the bread diets. But in the group treated with $CdCl_2$ we found a 5 fold increase in tissue Cd content (around 10 ng) compared to the bread diets (around 2 ng) (**Table 2**).

Measurement of luciferase activity in in vivo, ex vivo and in vitro

The sensitivity of the chosen animal model to $CdCl_2$ was earlier reported [5]. Whole body *in vivo* analysis of ER activity, done by measuring luciferase catalyzed photon emission at 0, 7, 14 and 21 days of treatment did not show major changes associated to the treatments (**Figure 2**), yet at the end of the study mice were euthanized, organs dissected and photon emission was measured by CCD camera in liver, small intestine, kidney and brain (**Figure 3A**). Significant changes were observed in kidney and brain: in kidney $CdCl_2$, but not bread diet treatments, induced a significant activation of ER (+126%), conversely in brain all treatments increased, at a different extent, photon emission ($CdCl_2$ +150%; wheat bread +286%; flax bread +110%) (**Figure 3B**). Due to the fact, that imaging is a 2D measurement, the luciferase expression in the inner most tissues cannot be understood. This led us to measure luciferase enzymatic activity in a broad range of tissues. **Figure 3C** shows that luciferase activity was modulated by the 21 day treatments in selected organs; as indicated by *ex vivo* measurement, Cd did not affect ER activity in liver and intestine, but we found increased luciferase expression in kidney of $CdCl_2$ treated mice (+150%); in brain luciferase increase was significant only in mice treated with wheat bread diet (+123%). Among the other organs studied, in skeletal muscle and WAT, $CdCl_2$ significantly increased ER activity (+167% and +133%, respectively); wheat bread, but not flax bread diet had a similar effect in the skeletal muscle (+133%), but not in WAT. In prostate, flax bread treatment clearly decreased luciferase content (-78%) and a non-significant trend to decrease was observed with $CdCl_2$ and wheat bread diet treatment.

Expression of endogenous target genes

To further evaluate ER transcriptional activity, we carried out a series of RTPCR studies to measure the effect of the different treatments on ER-α and on two genes known to be directly regulated by estrogens: progesterone receptor (PR) and prothymosine alpha (PTMA) [46]. In the prostate, ER-α mRNA content was significantly increased by all treatments ($CdCl_2$ +200%; wheat bread +500%; flax bread +270% *versus* controls); but was found unchanged in all the other tissues tested (brain, kidney, thymus and WAT). PR mRNA was significantly increased in prostate ($CdCl_2$ +750%; wheat bread +140%; flax bread +160%, versus controls), but decreased in WAT by wheat bread (-90% *versus* controls) and flax bread (-80% *versus* controls). No change was observed in the other organs taken into consideration. PTMA mRNA did not change significantly in all the tissues with the exception of the prostate where we observed a trend to an increase in the animals treated with $CdCl_2$ (+100% *versus* controls) (**Figure 4**).

Discussion

The present methodology was generated to investigate the effect of 21 day administration of Cd, as $CdCl_2$ or as an inherent bread contaminant, on ER signalling via ERE-containing promoters. The study for the first time, to the best of our knowledge, demonstrates that a cereal based diet or $CdCl_2$ may affect ER signalling in brain, prostate and WAT, in sexually developing immature male mice. We also show that the observed Cd related effects occur only at the molecular level and not at the anatomo-pathological level, due to various treatments.

Differential uptake of dietary Cd and $CdCl_2$

We found of particular interest the observation that Cd affects ER activity differentially with regard to the source of Cd; this may be in part due to the fact that, the diet interfered with Cd absorption [47]. $CdCl_2$ is more readily retained in kidney and liver, while the bread borne Cd is poorly concentrated in these tissues. The exposure of cadmium in the total 21 day intake in the three groups namely; in $CdCl_2$ (547 ng), wheat (776 ng) and flax (2131 ng) groups has accumulated in three important tissues together as 10.3 ng (4.4%), 2.3 ng (0.3%) and 2.5 ng (0.1%) respectively. A similar study describes the difference in bioaccumulation of Cd from a plant incorporated Cd containing diet to Cd added as a soluble salt to diet [48]. Cd in diet is although able to elicit some ER related changes, the non-accumulator scenario could be due

to the poor bioavailability and the matrix effect of diet masking the toxic effects of Cd. Food matrix may play a relevant role in these discrepancies as already reported in previous studies by our [5, 49] and other laboratories [50, 51].

Age and sex differences in Cd disposition

Significant accumulation of Cd from $CdCl_2$ treatment alone was found in the liver and kidneys of sexually immature male mice. But, in a similar study set up with adult ovariectomised female mice [5] treated with $CdCl_2$ or with flax bread diet showed significant increase in Cd content in the liver and small intestine and not in the kidneys. Interestingly, the basal Cd content measured in the female organs was significantly higher than in immature males: we speculate that this phenomenon could be due to differences in Cd metabolism and tissue distribution between immature males and adult females, or to the higher age, and therefore longer lifetime exposure, of females to dietary Cd.

In immature males, ER activity was affected by treatments in kidney, WAT, brain and skeletal muscle. In adult females, these organs were not affected [5]. But, ER activity was regulated in other organs like thymus. However, in WAT, Cd had similar effect in both animal models examined, possibly indicating that this tissue represents a novel, so far unknown target for Cd and Cd containing food. These gender specific effects observed may be in part a consequence of the differential metabolism and accumulation of the metal in the two animal models taken into consideration, but may also be ascribed to physiological differences. For instance, the differential effect in brain may be due to the fact that brain permeability to Cd in the young male mice is much higher than in adult females. The finding that Cd and/or Cd containing feed regulates ER activity in liver and kidney is in line with the known Cd effects [45, 52]. To be underlined is the differential response of ERs found in male and female mice. In males luciferase content was found to be induced by $CdCl_2$, while in females the effect was observed also in animals fed with wheat bread diet. However, when we analyzed the effect of the treatments on ER target genes such as PR, we found that PR content was decreased in males, but increased in females [5]. These dissimilarities suggest that the functional interaction of ERs with its target genes is significantly different in male and female mice.

The study of luciferase accumulation in prostate indicates that only flax bread diet induced significant changes, however, the quantitative measurement of ER-α mRNA showed that $CdCl_2$ and dietary Cd have a strong influence on the accumulation of this receptor in this organ. In view of the well reported proliferative action of the receptor, this change is most relevant and may point to a relevant toxic effect of Cd and dietary Cd in non adult males. Most interestingly prothymosine, a well-known marker of estrogen induced proliferation [46] was not significantly altered. Conversely PR mRNA was significantly modulated by all the treatments. Our findings are in line with previous reports indicating an involvement of this heavy metal in the development of prostate cancer [30, 53]. Altogether, WAT and prostate were identified as targets of Cd in sexually immature male mice and the rest of the observed EL effects are not consistent to a particular organ or treatment.

Sensitivity to low doses of Cd

Although Cd exposure at lower doses was shown to modulate molecular and functional parameters of estrogenicity, we have not observed any changes in necropsy between $CdCl_2$ treatment and controls. This indicates that despite differences in administration modalities, $CdCl_2$ as *per-oral* dose, dietary Cd from bread diets; the effects still seems to be less likely to be significant at this low dose by oral route. In fact, the no significant effects observed in our necropsy analysis could be due to the reason of "function of dose". In our experiments we used a low dose at the regulatory limits of human exposure. A study used diet with 50 mg/kg Cd concentration fed, to 7 week old male mice for 54 weeks and observed a "surprising" beneficial anticarcinogenic effect for Cd due to stimulation of metallothionenin and zinc in liver [54]. The authors [54] did not observe nephrosis at their concentration. Our experimental concentrations are beyond this limit to claim some possible detrimental effects. Our view was further supported by Hofer et al. 2009, in which $CdCl_2$ is given by oral route at a concentration of 0.05-4 mg/kg/bw for 3 days by *per-oral* dose and 0.4-9 mg/kg/bw for 4 weeks in drinking water, no changes in necropsy findings were observed in between

control and treatments, but some induction at the gene expression level [2]. This implies that the observed changes could have occurred only at the molecular level to induce/activate/repress ER and its related genes as an immediate effect due to treatments, but we are unable to speculate if the perturbation of the system during development might have consequences in the future life of the animal due to the pervasive nature of estrogen effects. The study demonstrates the sensitivity and specificity of the reporter mice coupled *in vivo* imaging methodology to study the EL effects of low doses of Cd as a dietary contaminant. The study also points to the difficulty of a univocal definition of toxicity for endocrine disrupters and illustrates the advantage of the use of reporter mice for the rapid identification of the tissues and organs where endocrine disruptors are active. This helps to focus on selected tissues where more in depth and longer toxicological analysis needs to be carried out to definitely establish the toxic potential of the compound of interest.

Significance to measure food safety

The major challenges for environmental and food toxicologists are the detection of contaminants that are generally present at very low concentrations embedded in a complex matrix and the evaluation of their cumulative effects due to accumulative process during long-term exposure. These contaminants are extremely difficult to screen and may potentially cause physiological disruption due to bioaccumulation. Current methodologies are capable to detect only adverse effects, hardly report small physiological changes and have a very limited predicting value. Moreover, the investigational tools are usually static in nature and detect toxicity in a snapshot of time; thus, providing only a partial view of the molecular mechanism underlying the toxic effects and classical tests in turn, use a large number of animals. Therefore, more effort should be focussed on generating appropriate model systems for the rapid, cost-effective and reproducible analysis of the overall effects of toxic contaminants on living organisms.

The concept of receptor mediated toxicity has driven the field of toxicology to carry out tests that are more predictable, leading to a thorough understanding of the mechanism based toxicity. In this methodology, reporter mice coupled bioluminescent in *vivo* imaging technology was shown as an experimental system to study the effects of low doses of contaminants in a complex food on estrogen receptor activity. *In vivo* imaging being cost effective, non-invasive, allowed to monitor the receptor dynamics in length of time (*tempora*l dimension) and provide a global view of the potential target organs of toxicity in all spectrum of body action of whole mouse (*spatial* dimension).

In conclusion, this study undertakes to analyse the Cd related EL effects and shows a imaging-based methodology to investigate the effect of extremely low concentrations of Cd embedded in food matrix and emphasizes the novel possibility to carry out long term exposure oriented studies of suspected chemicals in their inherent presence in the food chain or environment. The methodology further exemplifies the power of reporter systems-imaging technology offering a surrogate end point in a physiological set up and pro-sensitivity to acquire a global view of potential organs affected by the compound of interest.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Adriana Maggi, Center of Excellence on Neurodegenerative Diseases and Department of Pharmacological Sciences, University of Milan, Via Balzaretti, 9 I-20133 Milan, Italy. Tel: 0039.02.50318290; Fax: 003 9.02.50318287; E-mail: adriana.maggi@unimi.it

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