Letter

Influence of injection timing on severity of cadmium-induced testicular toxicity in mice

Katsumi Ohtani¹, Yukie Yanagiba², Atsushige Ashimori^{2,3}, Asuka Takeuchi^{2,3},
Naoko Takada³, Masako Togawa⁴, Tatsuya Hasegawa⁴, Masayuki Ikeda^{5,6}
and Nobuhiko Miura²

¹Division of Hazard Evaluation and Epidemiology, National Institute of Occupational Safety and Health Japan,
6-21-1, Nagao, Tama-ku, Kawasaki 214-8585, Japan

²Division of Health Effects Research, National Institute of Occupational Safety and Health Japan,
6-21-1, Nagao, Tama-ku, Kawasaki 214-8585, Japan

³Laboratory of Environmental Soil Science, School of Life and Environmental Science, Azabu University,
1-17-71 Fuchinobe, Chuo, Sagami-hara 252-5201, Japan

⁴Department of Environmental Biochemistry, Yamanashi Institute of Environmental Sciences,
5597-1, Kenmarubi, Kamiyoshida, Fujiyoshida, Yamanashi 403-0005, Japan

⁵Graduate School of Science and Engineering, University of Toyama, 3190, Gofuku, Toyama 930-8555, Japan

⁶Graduate School of Innovative Life Science, University of Toyama, 3190, Gofuku, Toyama 930-8555, Japan

(Received November 29, 2012; Accepted December 25, 2012)

ABSTRACT — Cadmium (Cd) is one of the endocrine disrupter and is a well-known testicular toxicant. Recently, we reported that Cd-induced mortality was markedly different by injection timing. In this report, we investigated whether severity of testicular toxicity was affected by injection timing of Cd. C57BL/6J mice (male, 7 w) were received single intraperitoneal injection of CdCl₂ (4.5 mg/kg) at zeitgeber time 6 (ZT6) or ZT18; these injection timings showed highest (ZT6) or lowest (ZT18) mortality in our previous study (Miura, 2012). After one week of the injection, several parameters for testicular toxicity such as epididymal sperm motility and numbers of sperm head both in cauda epididymidis and testis were measured. At ZT6 injection group, all parameters examined were significantly reduced compared to the control group. However, very interestingly, no significant changes were observed at ZT18 injection group. We obtained similar results by another experiment in which mice were received single subcutaneous injection of CdCl₂ (4 or 6 mg/kg) followed by measuring the parameters ten days after the injection. This diurnal variation was not contradictory to the result of the lethal toxicity which we showed earlier. Therefore, our results indicate that the testicular toxicity of Cd is also influenced by the injection timing.

Key words: Chronotoxicology, Cadmium chloride, Diurnal variation,
CASA (computer-assisted sperm analysis), Testicular toxicity, Circadian rhythm

INTRODUCTION

Many physiological and biochemical processes have been found to show 24-hr rhythmicity in laboratory animals and human beings. These processes are influenced by environmental factors such as light exposure, food ingestion, and environmental temperature (Aschoff, 1963; Ohdo, 2007, 2010). In consequence, the drug efficacies including their benefic and adverse effects show diurnal variation, since a number of biological reactions act for absorption, distribution, metabolism, and elimination of

drugs are under the control of circadian rhythm (Aschoff, 1963). This diurnal variation of drug actions has been recognized for over 50 years, developed as chronopharmacology including chronotherapy (Burioka *et al.*, 2010), and applied in clinical practice for treatment of variety of diseases such as cancer and diabetes (Focan, 2002; Bruguerolle, 2008). In toxicological field, however, studies for the diurnal variation of toxicity especially about metal compounds are performed insufficiently, although articles about several chemical compounds have been reported; for example, carbon tetrachloride (Bruckner

Correspondence: Nobuhiko Miura (E-mail: miuran@h.jniosh.go.jp)

et al., 2002), pesticides (Nicolau, 1982) and antibiotics (Beauchamp and Labrecque, 2007).

We have already reported that cadmium (Cd)-induced lethal toxicity was apparently varied by injection timing in a day (Miura *et al.*, 2012). Cd has serious and disadvantageous bioactivities such as endocrine disruption (Iavicoli *et al.*, 2009), immuno toxicity (Shippee *et al.*, 1983), and carcinogenicity (Joseph, 2009). Cd also attacks to testes and makes this organ a target for its toxicity as well as liver and kidney (Probst *et al.*, 1977; Maitani and Suzuki, 1986; Selypes *et al.*, 1992), further, disrupts the blood-testis barrier (Henson and Chedrese, 2004; Siu *et al.*, 2009). In this report, we tried to investigate the effect of injection timing on testicular toxicity induced by Cd injection.

MATERIALS AND METHODS

Chemicals and instrument

Cadmium chloride was purchased from Wako Pure Chemical (Osaka, Japan). Bovine serum albumin Fraction V (BSA) was purchased from Seikagaku Kogyo (Tokyo, Japan). Medium 199 was purchased from Invitrogen (Grand Island, NY, USA). Sterile saline was purchased from Ohtsuka Pharmaceutical (Tokyo, Japan). Semen analyzer (HTM-IVOS Ver. 12.1M) was the product of Hamilton Thorne Research (Beverly, MA, USA).

Experimental procedures

Male C57 BL/6J mice (5 weeks of age) were purchased from Nihon Clea (Clea Japan, Inc., Tokyo, Japan), and were kept in cages under standard conditions with controlled temperature ($24 \pm 1^\circ\text{C}$), humidity ($55 \pm 5\%$) and light (12:12 hr light/dark cycles, lights on at 08:00 hr). The lights were white fluorescent lamp, about 100-150 lux intensity at the level of cages. All animals had free access to sterilized commercial pellet diet (CE-2, Clea Japan, Inc., Tokyo, Japan), and sterilized filtered tap water. Mice were adapted for 14 days before experiments. The body weights were measured just before each injection and sacrifice.

To examine the influence of injection timing on severity of testicular toxicity, we performed experiments by two different injection routes. First, for an experiment of intraperitoneal (i.p.) injection, mice (7 weeks of age, $n = 4$) were received single i.p. injection of CdCl_2 (4.5 mg/kg) dissolved in saline at zeitgeber time 6 (ZT6) or ZT18. ZT means elapsed time from the lighting on. Control mice ($n = 2$) were received same volume (0.1 ml/10 g body weight) of saline at ZT6 or ZT18. Seven days after the injection, mice were sacrificed under ether anesthesia. Then, testes, epididymides and cau-

da epididymidis were separated and weighed followed by calculation of relative organ weights to body weights. The right cauda epididymidis was immediately analyzed for sperm motility (see "*Sperm motility*" in this section), and other reproductive organs were stocked in saline at 4°C until counting of the sperm head numbers (described in "*Sperm head numbers*"). Second, for an experiment of subcutaneous (s.c.) injection, mice (7 weeks of age, $n = 6-8$) were treated with single injection of CdCl_2 (4 mg/kg or 6 mg/kg) at ZT6 or ZT18. Control mice ($n = 4$) were prepared in both injection timing. Mice were sacrificed ten days after the injection. Testicular organs were collected and analyzed as described above. In our separate experiments, C57BL/6J mice showed clear hepatotoxicity in dose of more than 4.2 mg/kg of CdCl_2 treatment (estimated at 24 hr after the injection; data not shown).

These animal experiments were conducted under the guidelines for the care and use of laboratory animals set forth by our Institutional Animal Care and Use Committee of the National Institute of Occupational Safety and Health, Japan (JNIOH).

Sperm motility

Immediately after removal and weighting, the right cauda epididymis was minced with scissors to release sperms in 2 ml of Medium199 containing 0.5% BSA at 37°C . Sperm suspension was loaded into a capillary microslide (#HTR1099: 100 μm deep; VitroCom Inc., Mountain Lakes, NJ, USA) followed by analyzing the sperm motility with the system of computer-assisted sperm analysis (CASA) using HTM-IVOS (Hamilton Thorne Inc., Beverly, MA, USA) (Kato *et al.*, 2001). Two sperm motion parameters, motile percent (MP) and progressive percent (PP), were evaluated in each sample.

Sperm head numbers

The left cauda epididymis and the left testis were homogenized in saline. Sperm heads of each sample were stained with Hoechst-based dye (IDENT STAIN; Hamilton Thorne Inc.), dropped into a disposable counting chamber (CELL-VU; Millennium Sciences Corp., New York, NY, USA) and mounted on HTM-IVOS. Fluorescence of sperm heads under ultra-violet beam was monitored and counted in RAT-IDENT mode automatically. Data were expressed as total number of sperm heads per tissue sample (Ohtani *et al.*, 2004).

Statistical analysis

Data were analyzed by one-way ANOVA. Statistical significance of difference between control and Cd-treat-

ed groups was determined with Fisher's PLSD test. In all cases, $p < 0.05$ was considered statistically significant. For "i.p." injection group, the control mice injected at ZT6 and ZT18 ($n = 2$, respectively) were combined and expressed as one data (total $n = 4$).

RESULTS

We reported earlier that the Cd-induced lethal toxicity was different by injection timing; higher during daytime while lower during nighttime (Miura *et al.*, 2012). In order to investigate whether this difference is observed also in testicular toxicity, we treated CdCl_2 to mice at two different injection timing, ZT6 (daytime) or ZT18 (nighttime), which showed highest or lowest mortality in our previous study, respectively (Miura *et al.*, 2012). First, mice were received single i.p. injection of CdCl_2 (4.5 mg/kg) at ZT6 or ZT8, and were sacrificed seven days after the injection. Body weights and testicular organ weights (testes, epididymides, and cauda epididymis) showed no significant differences among control group, ZT6-injection group, or ZT18-injection group (Table 1). However, very interestingly, sperm head numbers of both cauda epididymidis and testis were significantly reduced at ZT6 injection while no reduction at ZT18 injection compared to the control (Fig. 1). Mammalian sperms mature while passing through the cauda epididymis (Seligman *et al.*, 1992) meanwhile sperms in testes are almost immature. Therefore, ZT6 injection reduced sperms in both of states at this injection dose. For sperm motility, ZT6 injection also significantly reduced two sperm motion parameters, motile percent (MP) and progressive percent (PP), which represented the sperm quality (Fig. 2). No such effects were observed at ZT18 injection same as the result of a sperm head numbers in Fig. 1. These results indicate that Cd injection at ZT6 introduces adverse effect on both quantity and quality of sperm.

Fig. 1. Effect of injection timing on sperm head numbers by i.p. injection. Male C57BL/6J mice ($n = 4$) were treated with single i.p. injection of CdCl_2 (4.5 mg/kg) at ZT6 or ZT18. Organ samples were collected seven days after the injection. The left cauda epididymis and the left testis were homogenized in saline followed by counting the sperm head numbers by HTM-IVOS. Control mice ($n = 2$) received saline at ZT6 or ZT8 were combined and expressed as one data (total $n = 4$). *Significantly different ($p < 0.05$) from control.

Table 1. Body weights and testicular organ weights of mice treated with CdCl_2 by i.p. injection.

Group name	Body weight	Testes	Epididymis	Cauda epididymis
Control [ZT6+ZT18] ($n = 2$, each)	23.7 ± 1.1	0.171 ± 0.009 (0.723 ± 0.067)	0.064 ± 0.008 (0.271 ± 0.033)	0.022 ± 0.003 (0.091 ± 0.012)
Cd [ZT6] ($n = 4$)	22.7 ± 0.9	0.175 ± 0.014 (0.774 ± 0.084)	0.061 ± 0.002 (0.268 ± 0.011)	0.023 ± 0.002 (0.099 ± 0.009)
Cd [ZT18] ($n = 4$)	22.1 ± 3.4	0.177 ± 0.007 (0.820 ± 0.170)	0.062 ± 0.002 (0.286 ± 0.059)	0.022 ± 0.001 (0.103 ± 0.021)

Male C57BL/6J mice were received single i.p. injection of CdCl_2 (4.5 mg/kg) at ZT6 or ZT18 followed by sacrificing seven days after the injection. The number (n) of animals in each group is shown in the column of "Group name". Weights are represented as gram. The relative ratio (%) of organ weights per body weights are shown in the parentheses under the organ weights (g). Values are means $\pm$ S.D.

Table 2. Body weights and testicular organ weights of mice treated with CdCl₂ by s.c injection.

Group name	Body weight	Testes	Epididymis	Cauda epididymis
Control [ZT6] (n = 4)	24.9 ± 2.2	0.188 ± 0.027 (0.757 ± 0.096)	0.062 ± 0.012 (0.250 ± 0.038)	0.019 ± 0.005 (0.075 ± 0.019)
Control [ZT18] (n = 4)	24.4 ± 0.6	0.173 ± 0.017 (0.709 ± 0.076)	0.066 ± 0.007 (0.271 ± 0.024)	0.025 ± 0.004 (0.102 ± 0.016)
Cd, 4 mg/kg [ZT6] (n = 6)	24.4 ± 0.9	0.177 ± 0.043 (0.725 ± 0.163)	0.066 ± 0.006 (0.271 ± 0.023)	0.022 ± 0.004 (0.090 ± 0.016)
Cd, 4 mg/kg [ZT18] (n = 6)	24.1 ± 1.2	0.188 ± 0.010 (0.783 ± 0.056)	0.058 ± 0.003 (0.242 ± 0.019)	0.021 ± 0.002 (0.087 ± 0.008)
Cd, 6 mg/kg [ZT6] (n = 8)	24.4 ± 1.0	0.185 ± 0.020 (0.759 ± 0.089)	0.060 ± 0.005 (0.246 ± 0.028)	0.019 ± 0.002 (0.079 ± 0.011)
Cd, 6 mg/kg [ZT18] (n = 6)	24.0 ± 1.1	0.176 ± 0.026 (0.731 ± 0.093)	0.059 ± 0.009 (0.244 ± 0.034)	0.020 ± 0.003 (0.083 ± 0.013)

Male C57BL/6J mice were received single s.c. injection of CdCl₂ (4 mg/kg or 6 mg/kg) at ZT6 or ZT18 followed by sacrificing ten days after the injection. The number (n) of animals in each group is shown in the column of "Group name". Weights are represented as gram. The relative ratio (%) of organ weights per body weights are shown in the parentheses under the organ weights (g). Values are means ± S.D.

Fig. 2. Effect of injection timing on sperm motility by i.p. injection. Organ samples were collected from same mice as described in Fig. 1. Immediately after removal of organ samples from mouse, the right cauda epididymis was minced in 2 ml of Medium199 containing 0.5% BSA. The sperm motility was analyzed by HTM-IVOS. Two sperm motion parameters, motile percent (MP) and progressive percent (PP), were examined. *Significantly different ($p < 0.05$) from control.

By intraperitoneal injection, however, it is possible that Cd attacks directly to reproductive organs like testes and cauda epididymidis. Therefore, we performed examination by another injection route, subcutaneous injection, and set up longer period to dissection since Cd was thought to act through a blood flow. Mice were received single s.c. injection of CdCl₂ (4 mg/kg or 6 mg/kg) at ZT6 or ZT18 followed by sacrificing at ten days after the injection. The body weights and reproductive-related organ weights were not significantly different among control and treatment groups, same as the result of i.p. injection experiment (Table 2). The sperm head numbers in 4 mg/kg of CdCl₂ treatment group, however, were significantly reduced by ZT6 injection but not by ZT18 injection (Fig. 3). In 6 mg/kg treatment group, the numbers of sperm heads were reduced at both injection timing compared to control group. For sperm motility, ZT6 injection significantly reduced the two sperm motion parameters in 6 mg/kg treatment group, although no change was observed in 4 mg/kg treatment group at both injection timings (Fig. 4). Our current data clearly indicated that the difference of susceptibility to Cd by injection timing was observed also in testicular toxicity same as the result of Cd-induced mortality which we reported earlier.

DISCUSSION

In this study, the difference of injection timing (ZT6 or ZT18) clearly altered the severity of Cd-induced testicular toxicity evaluated by reduction of both sperm head numbers and sperm motility as indexes; ZT6 injection was more toxic compared to ZT18 injection. This diurnal

Diurnal variation of cadmium-induced testicular toxicity in mice

Fig. 3. Effect of injection timing on sperm head numbers by s.c. injection. Male C57BL/6J mice ($n = 6-8$) were treated with single s.c. injection of CdCl_2 (4 mg/kg or 6 mg/kg) at ZT6 or ZT18. Control mice ($n = 4$) were prepared in both injection timing. Mice were sacrificed ten days after the injection. Sperm head numbers were counted as same as Fig. 1. *Significantly different ($p < 0.05$) from control.

Fig. 4. Effect of injection timing on sperm motility by s.c. injection. Organ samples were collected from same mice as described in Fig. 3. The sperm mobility was analyzed as same as Fig. 2. *Significantly different ($p < 0.05$) from control.

nal variation was not contradictory to the result of the Cd-induced mortality which we showed earlier (Miura *et al.*, 2012), indicating the Cd-induced daily fluctuation was observed not only in mortality but also in testicular toxicity. In our recent examination, several metal compounds other than Cd also produced the diurnal variation of mortality (manuscript in preparation). Therefore, this phenomenon is thought to be common to some extent among metals, although the examination about testicular toxicity will be required.

Investigations of levels of Cd in testicular organs and levels of biological defense factors are needed. If Cd accumulation levels in testes or cauda epididymis are different by injection timing, transporters like ZIP8, ZIP14 and DMT1 (Kim *et al.*, 2007; Girijashanker *et al.*, 2008),

which involve in Cd transport into these organs, may contribute to the diurnal variation. Induction level of metallothionein, a well known and important defense factor against Cd toxicity, should also be considered (Siu *et al.*, 2009). Further, we have to get information about differences of oxidative status and levels of anti-oxidative factors in the reproductive organs after Cd injection at daytime or nighttime, because Cd is known to induce oxidative stress in testes (Ardais *et al.*, 2008; Ji *et al.*, 2012). Examinations from these points including pathological analyses are in progress.

The sperm progressive parameter used in this study (as PP) is thought to be important because this index relate closely to fertility (Ban *et al.*, 2001; Kato *et al.*, 2001). Therefore, the difference in exposure timing of Cd may cause not only testicular toxicity but reproductive impairment. It is necessary to clarify whether the injection tim-

ing influences reproductive at low dose of Cd, since Cd acts as endocrine disruptor by low-dose repeated administration (Henson and Chedrese, 2004; Siu *et al.*, 2009). Although it is difficult to extrapolate our animal data to human beings at present, we believe this viewpoint, the susceptibility difference by injection timing, should be introduced into toxicology as “chronotoxicology” becoming indispensable in the future toxicology.

ACKNOWLEDGMENTS

We thank Dr. Masaya Takahashi and Dr. Yasutaka Ogawa, National institute of Occupational Safety and Health, Japan (JNIOSH) for helpful advice and coordinating of our team. This work was supported by grant-in-aid for project research (P21-01) from National Institute of Occupational Safety and Health, Japan (JNIOSH).

REFERENCES

- Ardaïs, A.P., Santos, F.W. and Nogueira, C.W. (2008): Ebselen attenuates cadmium-induced testicular damage in mice. *J. Appl. Toxicol.*, **28**, 322-328.
- Aschoff, J. (1963): Comparative physiology: diurnal rhythms. *Annu. Rev. Physiol.*, **25**, 581-600.
- Ban, Y., Naya, M., Nishimura, T., Kaneto, M., Kishi, K., Inoue, T., Yoshizaki, H. and Ooshima, Y. (2001): Collaborative study on rat sperm motion analysis using CellSoft Series 4000 semen analyzer. *J. Toxicol. Sci.*, **26**, 9-24.
- Beauchamp, D. and Labrecque, G. (2007): Chronobiology and chronotoxicology of antibiotics and aminoglycosides. *Adv. Drug Deliv. Rev.*, **59**, 896-903.
- Bruckner, J.V., Ramanathan, R., Lee, K.M. and Muralidhara, S. (2002): Mechanisms of circadian rhythmicity of carbon tetrachloride hepatotoxicity. *J. Pharmacol. Exp. Ther.*, **300**, 273-281.
- Bruguerolle, B. (2008): Clinical chronopharmacology in the elderly. *Chronobiol. Int.*, **25**, 1-15.
- Burioka, N., Fukuoka, Y., Koyanagi, S., Miyata, M., Takata, M., Chikumi, H., Takane, H., Watanabe, M., Endo, M., Sako, T., Suyama, H., Ohdo, S. and Shimizu, E. (2010): Asthma: Chronopharmacotherapy and the molecular clock. *Adv. Drug Deliv. Rev.*, **62**, 946-955.
- Focan, C. (2002): Chronobiological concepts underlying the chemotherapy of human lung cancer. *Chronobiol. Int.*, **19**, 253-273.
- Girijashanker, K., He, L., Soleimani, M., Reed, J.M., Li, H., Liu, Z., Wang, B., Dalton, T.P. and Nebert, D.W. (2008): Slc39a14 gene encodes ZIP14, a metal/bicarbonate symporter: similarities to the ZIP8 transporter. *Mol. Pharmacol.*, **73**, 1413-1423.
- Henson, M.C. and Chedrese, P.J. (2004): Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction. *Exp. Biol. Med.*, **229**, 383-392.
- Iavicoli, I., Fontana, L. and Bergamaschi, A. (2009): The effects of metals as endocrine disruptors. *J. Toxicol. Environ. Health B Crit. Rev.*, **12**, 206-223.
- Ji, Y.L., Wang, H., Meng, C., Zhao, X.F., Zhang, C., Zhang, Y., Zhao, M., Chen, Y.H., Meng, X.H. and Xu, D.X. (2012): Melatonin alleviates cadmium-induced cellular stress and germ cell apoptosis in testes. *J. Pineal. Res.*, **52**, 71-79.
- Joseph, P. (2009): Mechanisms of cadmium carcinogenesis. *Toxicol. Appl. Pharmacol.*, **238**, 272-279.
- Kato, M., Fukunishi, K., Ikegawa, S., Higuchi, H., Sato, M., Horimoto, M. and Ito, S. (2001): Overview of studies on rat sperm motion analysis using a Hamilton-Thorne Sperm Analyzer—collaborative working study. *J. Toxicol. Sci.*, **26**, 285-297.
- Kim, D.W., Kim, K.Y., Choi, B.S., Youn, P., Ryu, D.Y., Klaassen, C.D. and Park, J.D. (2007): Regulation of metal transporters by dietary iron, and the relationship between body iron levels and cadmium uptake. *Arch. Toxicol.*, **81**, 327-334.
- Maitani, T. and Suzuki, K.T. (1986): Effect of cadmium on essential metal concentrations in testis, liver and kidney of five inbred strains of mice. *Toxicology*, **42**, 121-130.
- Miura, N., Yanagiba, Y., Ohtani, K., Mita, M., Togawa, M. and Hasegawa, T. (2012): Diurnal variation of cadmium-induced mortality in mice. *J. Toxicol. Sci.*, **37**, 191-196.
- Nicolau, G.Y. (1982): Circadian rhythms of RNA, DNA and protein content in the rat thyroid, adrenal and testis in chronic pesticide exposure. I. Effects of a fungicide (Mancozeb). *Endocrinologie*, **20**, 249-257.
- Ohdo, S. (2007): Chronopharmacology focused on biological clock. *Drug Metab. Pharmacokinet.*, **22**, 3-14.
- Ohdo, S. (2010): Chronopharmaceutics: pharmaceuticals focused on biological rhythm. *Biol. Pharm. Bull.*, **33**, 159-167.
- Ohtani, K., Yamazaki, S., Kubota, H., Miyagawa, M. and Saegusa, J. (2004): Comparative investigation of several sperm analysis methods for evaluation of spermatotoxicity of industrial chemical: 2-bromopropane as an example. *Ind. Health*, **42**, 219-225.
- Probst, G.S., Bousquet, W.F. and Miya, T.S. (1977): Correlation of hepatic metallothionein concentrations with acute cadmium toxicity in the mouse. *Toxicol. Appl. Pharmacol.*, **39**, 61-69.
- Seligman, J., Kosower, N.S. and Shalgi, R. (1992): Effects of caput ligation on rat sperm and epididymis: protein thiols and fertilizing ability. *Biol. Reprod.*, **46**, 301-308.
- Selyes, A., Serenyi, P., Boldog, I., Bokros, F. and Takacs, S. (1992): Acute and “long-term” genotoxic effects of CdCl₂ on testes of mice. *J. Toxicol. Environ. Health*, **36**, 401-409.
- Shippee, R.L., Burgess, D.H., Ciavarrà, R.P., DiCapua, R.A. and Stake, P.E. (1983): Cadmium-induced suppression of the primary immune response and acute toxicity in mice: differential interaction of zinc. *Toxicol. Appl. Pharmacol.*, **71**, 303-306.
- Siu, E.R., Mruk, D.D., Porto, C.S. and Cheng, C.Y. (2009): Cadmium-induced testicular injury. *Toxicol. Appl. Pharmacol.*, **238**, 240-249.