

Decreased Gene Expression of Testicular Cell-Specific Proteins in Cadmium-Induced Acute Testicular Toxicity

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Cadmium salts induce severe acute testicular necrosis in rodents. We assessed the expression levels of the genes encoding the follicle-stimulating hormone receptor, luteinizing hormone receptor, testis-specific histone 2B, and transition proteins 1 and 2, which are preferentially expressed in Sertoli cells, Leydig cells, spermatocytes, and spermatids, respectively, by reverse transcription (RT)-PCR using total RNA prepared from the whole testes of cadmium chloride (Cd)-administered rats. Sprague Dawley rats at 3, 7, and 12 weeks of age were singly and subcutaneously injected with Cd at doses of 1, 2.5, 5, 10, 15 or 20 $\mu\text{mol/kg}$. The expression levels of all genes tested were significantly decreased at doses of over 15 $\mu\text{mol/kg}$ (3-week-old rats) or over 10 $\mu\text{mol/kg}$ (7- and 12-week-old rats) 96 hr after injection. Histopathological study showed that these dosages of Cd resulted in extensive disruption of the seminiferous tubules and necrosis of testicular cells, while administration of Cd at a dose of 5 $\mu\text{mol/kg}$ in 7- and 12-week-old rats resulted in only partial degeneration of seminiferous tubules and testicular cells. Therefore, their reduced gene expression is likely to serve as an indicator of Cd-induced testicular necrosis accompanied by cell death. In addition, the susceptibility to Cd-induced testicular damage and decreased gene expression was higher in mature (7- and 12-week-old) rats than in immature (3-week-old) rats.

Key words — cadmium, testis, toxicity, necrosis, gene expression

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INTRODUCTION

The testicular toxicity of cadmium salts (Cd) has been demonstrated in many animal species.¹⁾ For example, total necrosis in major component cell populations of the testis is observed within 24–48 hr after subcutaneous single injection of Cd at a dose of approximately 20 $\mu\text{mol/kg}$ to rats.¹⁾

Various direct effects of Cd on cells derived from a wide variety of tissues have been proposed on the basis of many *in vitro* approaches.^{2–4)} However, the levels of Cd accumulated in the testis are relatively low compared with those in many other tissues such as the liver after Cd-administration.^{5,6)} Therefore, the precise process of Cd-induced testicular necrosis *in vivo* remains to be defined, including whether the testicular necrosis results from the direct action of Cd to testicular cells. At present, it has generally been thought that disruption of the blood-testis barrier occurring before the onset of testicular necrosis changes the physiological environment within the testis, and that these changes consequently induce damage in testicular cells.^{4,7–9)}

It seems to be an effective approach to identify genes and proteins undergoing expressional changes in testicular cells in response to Cd-administration as a means of clarifying the precise mechanism of Cd-induced testicular toxicity. Changes in the expression of some testicular genes such as p53,^{10,11)} c-jun,¹¹⁾ luteinizing hormone receptor (LHR),¹²⁾ heme oxygenase-1,¹³⁾ and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)¹⁴⁾ during the development of Cd-induced testicular necrosis have already been reported. In addition, the cDNA microarray assay was performed to determine the testicular profiles of gene expression in mice administered with

Table 1. Primers Used for PCR

Gene	Primer
FSHR	5'-AGGGCCAGGTCAACATACCGCTT-3' (forward)
	5'-TGGTGAGCAGGTCACATCAAC-3' (reverse)
LHR	5'-GCATCTGTAAACACAGGCATCCG-3' (forward)
	5'-CGATGAGCGTCTGAATGGACT-3' (reverse)
TH2B	5'-GTCGGCAAAGGGGACTACTATTTC C-3' (forward)
	5'-TTGGAGCTGGTGTACTTGGTGAC-3' (reverse)
TP1	5'-CCAGCCGCAAACATAAAGACTCATGG-3' (forward)
	5'-AGTCATTGCCGCATTACAAGTGGG-3' (reverse)
TP2	5'-AGGAAAGGTGAGCAAGAGAAAGGCG-3' (forward)
	5'-CATTCCCCTAGTGATGGCTATCTCC-3' (reverse)
GAPDH	5'-TGTGAACGGATTTGGCCGTA-3' (forward)
	5'-TCGCTCCTGGAAGATGGTGA-3' (reverse)

Cd at a non-toxic dose.¹⁵⁾ However, the amount of information still not be sufficient to understand the precise mechanism of Cd-induced testicular toxicity.

In the present study, we noticed expressional changes in genes that are expressed specifically or preferentially in the major testicular component cells in response to the degree of Cd-induced testicular cell damage. The genes examined were LHR for Leydig cells,¹⁶⁾ the follicle-stimulating hormone receptor (FSHR) for Sertoli cells,¹⁷⁾ testis-specific histone 2B (TH2B) for spermatocytes,^{18, 19)} and transition protein 1 (TP1)^{19, 20)} and TP2^{19, 21)} for round spermatids. The decrement of the LHR gene expression has already been reported in the testis of rats administered with Cd,¹²⁾ while the expressional changes of the other genes have not yet been reported.

MATERIALS AND METHODS

Animals and Treatments—Male Sprague-Dawley (SD) rats were obtained from Japan SLC Inc. (Hamamatsu, Japan). The animals were housed under controlled environmental conditions with free access to water and food and with a 12-hr light/dark cycle. Cadmium chloride (Cd) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and dissolved in sterile saline (0.9% NaCl). The animals (3-, 7-, and 12-week-old) were subcutaneously administered a single dose of Cd at doses of 1, 2.5, 5, 10, 15, or 20 $\mu\text{mol/kg}$. Control animals received saline only (0 $\mu\text{mol/kg}$). The animals were sacrificed by decapitation 96 hr after treatment, and the testes were obtained. Each group contained three

animals. Experimental protocols were approved by the Animal Experimentation Ethical Committee at the University of Shizuoka.

RNA Preparation and RT-PCR—Testes were rapidly removed from Cd-untreated (control) and treated rats, quickly frozen in liquid nitrogen, and stored at -80°C until being processed for RNA preparation, as described below. Total RNA was prepared from the testes with ISOGEN (Nippon-Gene, Toyama, Japan) and used to determine the expression levels of each gene. Briefly, a portion (4 μg) of the total RNA was converted into cDNA using polyd(N)₆ primer (Pharmacia Biotech, Piscataway, NJ, U.S.A.) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) in an reverse transcription (RT)-reaction mixture (20 μl). PCR was performed using 0.8 μl of the RT-reaction mixture, 0.5 μM of each primer (forward and reverse primers), and AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT, U.S.A.) in 25 μl of total reaction mixture. The primer sets used are shown in Table 1. The amplification protocol consisted of 20–35 cycles of denaturation for 1 min at 95°C , annealing for 1 min at 60°C , and extension for 2 min at 72°C for each gene. The PCR products were visualized by ethidium bromide staining under UV light following electrophoresis on a 2% agarose gel. The amounts of the separated PCR products were densitometrically determined with a computer using NIH Image software version 1.62.

Testicular Histopathology—Testes fixed in 10% neutral formalin were processed by standard histopathological techniques and stained with hematoxylin and eosin for light microscopic examination.

Statistical Analyses—Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Dunnett's test, using a software, JMP version 7 (SAS Institute Japan, Inc., Tokyo, Japan).

RESULTS AND DISCUSSION

Changes in the testicular gene expression levels of FSHR, LHR, TH2B, TP1, and TP2 were evaluated 96 hr after Cd-administration with the 3-week-old rats at doses of 5, 10, 15, and 20 $\mu\text{mol/kg}$, with the 7-week-old rats at doses of 2.5, 5, 10, and 15 $\mu\text{mol/kg}$, and with the 12-week-old rats at doses of 1, 2.5, 5, and 10 $\mu\text{mol/kg}$ (Fig. 1). Cd-administration at doses of 15 and 20 $\mu\text{mol/kg}$ resulted in a marked decrease in the gene expression levels of LHR, FSHR, and TH2B in testicular immature (3-week-old) rats. The gene expression of TP1 and TP2 was not detected in the rats with or without Cd-administration. On the other hand, markedly reduced expression of all the genes tested was observed at doses over 10 $\mu\text{mol/kg}$ in the mature (7- and 12-week-old) rats. Statistical analysis indicated that these decreased levels were significantly different from those of the control or at other doses.

The magnitude of testicular tissue injury at 96 hr after Cd-administration was histopathologically examined (Fig. 2). In Cd-treated 3-week-old rats, extensive injury with disruption of the seminiferous tubules, necrosis of testicular cells (spermatogonia, spermatocytes, and Sertoli cells), and vacuolar degeneration of Leydig cells were observed at doses over 15 $\mu\text{mol/kg}$, but not at less than 10 $\mu\text{mol/kg}$. Sperm cells and spermatozoa did not appear in the rats administered with or without Cd. In the Cd-administered 7- and 12-week-old rats, extensive disruption of the seminiferous tubules and necrosis of testicular cells (spermatogonia, spermatocytes, sperm cells, spermatozoa, Sertoli cells, and Leydig cells) were observed at doses over 10 $\mu\text{mol/kg}$. The administration at 5 $\mu\text{mol/kg}$ led to partial degeneration in seminiferous tubules and testicular cells, although the magnitude of the injury was far less than at doses over 10 $\mu\text{mol/kg}$. In addition, the injury levels at a dose of 5 $\mu\text{mol/kg}$ in 12-week-old rats appeared to be more advanced than those at the same dose in 7-week-old rats. Spermatozoa were observed in the epididymis of 7- and 12-week-old Cd-administered rats at a dose of 5 $\mu\text{mol/kg}$ (data

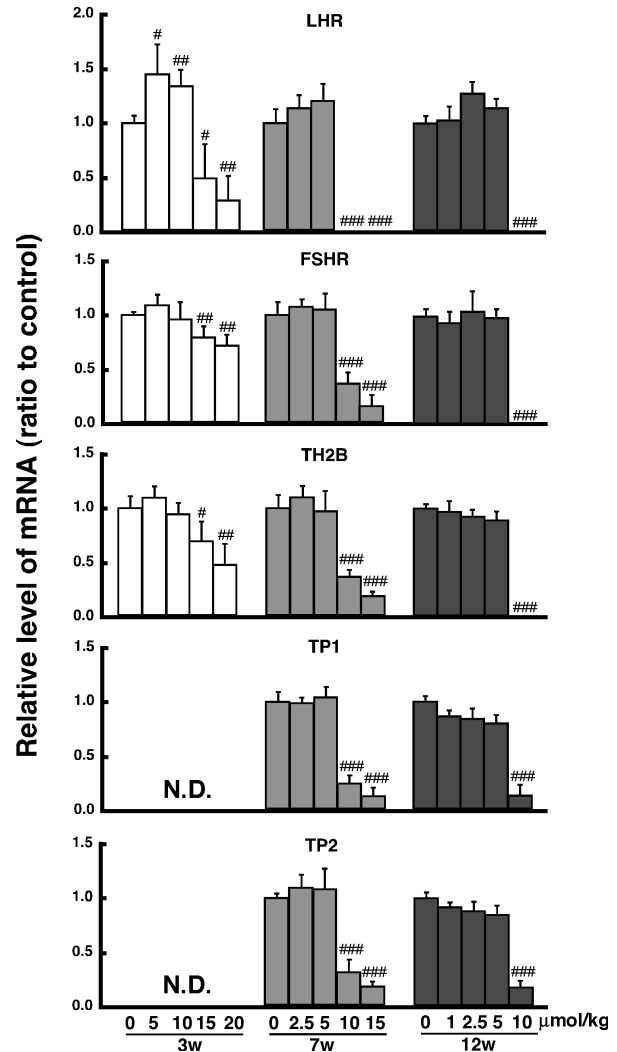


Fig. 1. Effects of Age on Cd-induced Toxicity and of Dose of Cd on the Gene Expression of Testicular Cell-specific Proteins

Cd was administered to the 3-, 7-, and 12-week-old rats at the indicated doses. As a control, the age-matched rats were given saline alone. The RT-PCR products using the testicular RNA from the rats 96 hr after each treatment were separated by agarose gel electrophoresis. The amounts of RT-PCR products were densitometrically determined with a computer using NIH Image software version 1.62, and are shown as a ratio to the corresponding controls. Values are mean \pm S.D. ($n = 3$ for each group). # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, significant difference from the corresponding control.

not shown).

The present study indicated that all of the expression levels of the genes tested responsible for testicular cell-specific proteins, LHR, FSHR, TH2B, and TP1/TP2, were significantly decreased at doses over 15 $\mu\text{mol/kg}$ (3-week-old rats) or over 10 $\mu\text{mol/kg}$ (7- and 12-week-old rats). As for the LHR gene among the genes we tested, Gunnars-son *et al.* have already reported the decrement of its expression in mature rats administered at a dose

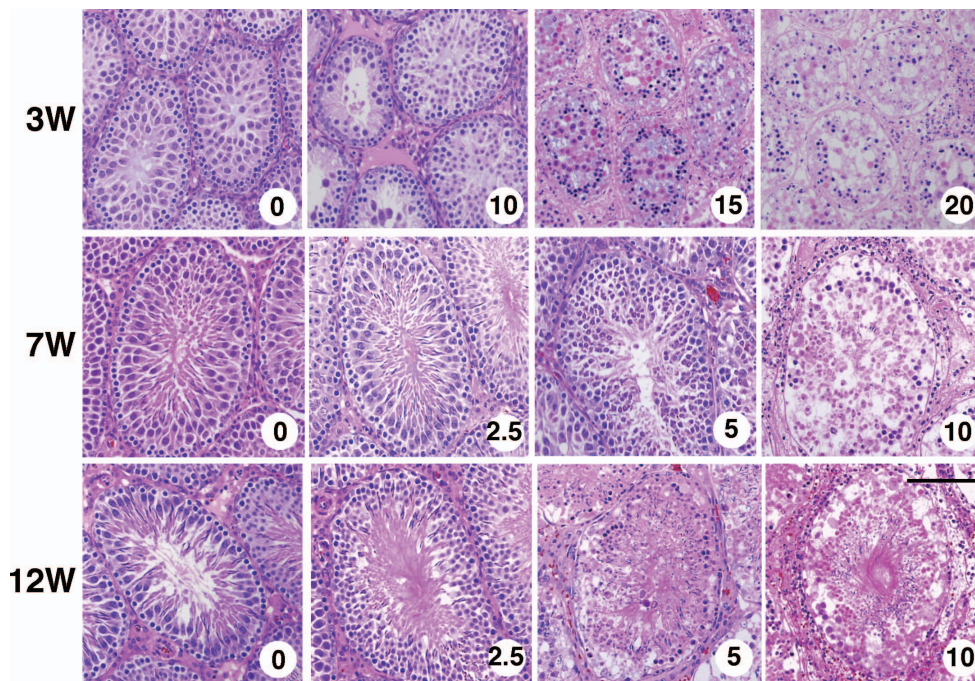


Fig. 2. Histopathology of the Testes 96 hr after Treatment with Various Doses of Cd to the 3-, 7-, and 12-week-old Rats
Numbers in white circles indicate the given doses ($\mu\text{mol/kg}$). Bar = 100 μm . Similar results were obtained in the other two rats per group.

of 10 $\mu\text{mol/kg}$ but not 5 $\mu\text{mol/kg}$,¹²⁾ identical to our present results. However, they did not make mention of the association between this decrement and the testicular damage. Our histopathological study showed that the dosages of Cd leading to significantly reduced expression of the genes tested; *i.e.*, over 15 $\mu\text{mol/kg}$ in 3-week-old rats and over 10 $\mu\text{mol/kg}$ in 7- and 12-week-old rats, resulted in extensive disruption of the seminiferous tubules and necrosis of testicular cells, while administration of Cd at a dose of 5 $\mu\text{mol/kg}$ in 7- and 12-week-old rats resulted in only partial degeneration of seminiferous tubules and testicular cells. Therefore, the significantly decreased expression of the genes tested was in accordance with Cd-induced severe testicular necrosis but not with partial degeneration in seminiferous tubules and testicular cells. In damage levels as found at a dose of 5 $\mu\text{mol/kg}$ (7- and 12-week-old rats), most testicular cells might have enough vital activity to sustain the expression of these genes. It will be important to verify this hypothesis and to investigate the effects of Cd at such doses on testicular cells.

In addition, we found that at the gene expression level the susceptibility to Cd-induced testicular damages is higher in testicular mature (7- and 12-week-old) rats than in immature (3-week-old) rats; these findings are similar to those previously

obtained at the histopathological level, as reported by Wong and Klaassen.²²⁾ Moreover, the expression levels of the tested genes were drastically decreased at a dose of 15–20 $\mu\text{mol/kg}$ in 3-week-old rats and at a dose of 10 $\mu\text{mol/kg}$ in 7- and 12-week-old rats, suggesting that the threshold doses of Cd for whole testicular damage fall in a very narrow range; *e.g.*, 10–15 or 10–20 $\mu\text{mol/kg}$ in 3-week-old rats and 5–10 $\mu\text{mol/kg}$ in 7- and 12-week-old rats. Therefore, identification of factors defining the age difference and narrow threshold would provide a clue to determining the mechanism for Cd-induced testicular toxicity.

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