Letter to the Editor

Effect of Cadmium on Rat Leydig Cell Testosterone Production and DNA Integrity *in vitro*[*]

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Cadmium (Cd) is an elemental heavy metal with widely recognized toxicity. Its long-term use in industrial processes and daily activities has caused alarming levels of Cd contamination in the natural environment. According to the estimates by the Agency of Toxic Substances and Disease Registry in the US, 25 000 to 30 000 metric tons of Cd is annually released to the environment[1]. Results of previous studies have demonstrated that several organs are targets of Cd, but the most important of these targeted organs may be the testes. Cadmium can cause the apoptosis of testis cells and modify the expression of carcinogenesis-related genes to cause cancer in the testes[2], interfere with the male reproductive endocrine functions[3], or even alter gene expression at non-toxic doses[4]. To date, the mechanism underlying the reproductive toxicity caused by Cd in male animals remains unclear. Here, the effect of Cd on primary cultures of Leydig cells was investigated *in vitro*.

Leydig Cell Isolation The rat testes were decapsulated under aseptic conditions. The Leydig cells were then isolated using collagenase and purified using a discontinuous Percoll gradient, as described by Lee et al.[5] and Kerr[6].

Identification of Vitality and Purity The viability of purified Leydig cells was determined by trypan blue exclusion. A 100 µL cell suspension was mixed with 5 µL 1% trypan blue (in PBS, pH 7.4) and the stained cells were counted using a hemocytometer after 2 min of staining. Leydig cells with ≥95% viability were used for further experiments.

The purity of the cultured Leydig cells was assessed using the modified Wiebe method[10]. A 10 µL cell suspension was smeared on slides and dried at room temperature. The smear was fully covered with the substrate solution, incubated at 22 °C for 90 min, washed with deionized water, and dried. The slides were examined under a microscope, and the dark blue cells (which were considered Leydig cells) were counted in 20 fields-of-view. The substrate solution contained 0.1% bovine serum albumin, 1.5 mmol/L nicotinamide adenine dinucleotide (NAD^+), 0.25 mmol/L nitroblue tetrazolium (NBT), and 0.2 mmol/L dehydroepiandrosterone (dissolved in minute *N*,*N*-dimethylformamide) in Ca^{2+}- and Mg^{2+}-free PBS (pH 7.4).

Cadmium Treatment and Viability Test Cadmium chloride (5 mmol/L) was diluted in serum-free M199 media to final concentrations of 5, 10, 25, 50, and 100 µmol/L. The control group was not treated with Cd.

Trypan blue method: Leydig cells in 24-well plates were treated with Cd at final concentrations of 5, 10, 25, and 100 µmol/L for 24 h. After removing the culture media, the cells were stained with 1% trypan blue and examined under an inverted microscope. Eight wells were assayed for each group, and more than 500 cells were counted in each well.

Methyl thiazolyl tetrazolium (MTT) method: Leydig cells were cultured in 96-well plates. Blank control wells without any cells were set-up, which contained the culture medium, MTT, and dimethyl sulfoxide (DMSO). The remaining wells were seeded with Leydig cells, which were treated with cadmium chloride at final concentrations of 5, 10, 25, and 100 µmol/L. After 24 h treatment, 20 µL of MTT (5 g/L in PBS) was added to each well, and the mixtures were incubated for 4 h. Subsequently, 150 µL DMSO was added to each well, which was followed by shaking for 20 min. The optical density of the cell suspensions was measured as the absorbance at 490 nm. Each treatment was repeated eight times.

Determination of Testosterone Secretion Human chorionic gonadotropin was added to the culture medium at a final concentration of 10 U/mL. The cells were treated with final Cd concentrations of 5, 10, 25, and 100 µmol/L for 24 h. Testosterone secretion was determined via a radioimmunoassay (RIA), according to the protocol recommended by the manufacturer (Shanghai Bioproduct Institute)

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using an 8N-695A automated radio-immune γ counter. I^{125} was used as the isotope. Each treatment was repeated eight times.

3β-HSD Activity Assay The enzymatic activity for testosterone synthesis was determined using the 3β-hydroxylsteroid dehydrogenase (3β-HSD) staining method. Briefly, Leydig cells were incubated with the substrate solution at 34 °C for 2 h. The number of dark-blue 3β-HSD-positive cells was estimated. The ratio of positive cells (number of positive cells/total number of cells × 100%) was used to represent the 3β-HSD activity. Each treatment was repeated six times. The substrate solution contained 150 mg NAD^+ and 25 mg 5β-androstane-3β-ol-17-one in 10 mL DMF, with 50 mg NBT dissolved in a small amount of PBS; these components were mixed with PBS to a total volume of 100 mL.

DNA Damage Assay After Cd treatment (similar to the viability test), the DNA single-strand breakage was assessed by single-cell gel electrophoresis (SCGE). Based on the method by Singh[8], the comet rate and migration distance were examined using a fluorescence microscope with a 515 nm to 560 nm excitation filter and a 590 nm emission filter.

Data Analysis Data were processed in Microsoft Excel 2000, including the general purpose analysis, graphing, database format conversion, and linear regression analysis. The cell viability, testosterone secretion, and 3β-HSD activity results were analyzed by one-way ANOVA using SPSS (version 19). The data on DNA damage were analyzed by the *t*-test and the Mantel-Haenszel χ^2 test[11] using SAS for Windows (version 6.12). All data were presented as the mean±standard deviation (SD), unless stated otherwise.

Leydig Cell Isolation and Identification The Wiebe stained cells are shown in Figure 1-A, with the rat Leydig cells stained dark blue.

Leydig Cell Viability after Cd Treatment The results of the MTT assay and trypan blue method showed that all groups exposed to Cd for 24 h had significantly lower cell viability than the control group (P<0.05). Differences among the groups exposed to different Cd concentrations were all statistically significant (P<0.05). Results of the linear regression analysis indicated that Cd concentrations less than 50 μmol/L had a significant effect on the cell viability (trypan blue method: $y=94.89e^{-0.0625x}$, $R^2=0.9897$; MTT method: $y=111.51e^{-0.0543x}$, $R^2=0.9884$) (Figure 2-A).

Leydig Cell Function after Cd Exposure Testosterone Secretion The RIA results showed that all Cd-exposed groups had significantly lower testosterone levels than the control group (P<0.05). The testosterone level was inversely affected by the Cd concentration. The assay demonstrated that the effect of Cd concentrations less than 10 μmol/L ($y=-3.027x+39.348$, $R^2=0.9773$) on testosterone secretion was greater than that of concentrations higher than 25 μmol/L ($y=-0.0531x+6.835$, $R^2=0.9844$) (Figure 2-B).

3β-HSD Activity in Leydig Cells The percentage of dark blue-stained Leydig cells (3β-HSD-positive cells) and the depth of staining were inversely correlated with the Cd dosage (Figure 1-B and Figure 2-C). The 3β-HSD-positive cell rates in the Cd-exposed groups were markedly lower than those in the control group (P<0.05) in a dose-dependent manner. The assay showed that Cd concentrations less than 10 μmol/L ($y=-5.698x+59.393$, $R^2=0.8697$) had a greater effect on 3β-HSD activity, as compared with the concentrations higher than 25 μmol/L ($y=-0.0517x+4.925$, $R^2=0.8998$).

Leydig Cell DNA Damage upon Cd Exposure The observed comet rates are summarized in Table 1. The Leydig cell comet rates were positively correlated with the Cd dose. The Mantel–Haenszel χ^2 test[11] demonstrated that Cd exposure significantly changed the comet rate (P<0.01). The DNA migration rates were likewise positively correlated with the Cd dosage. Significant differences were observed among the Cd-exposed groups (P<0.05), thereby suggesting that Cd exposure causes DNA damage (Figure 2-D and Figure 3).

Table 1 Effect of Cadmium on the DNA Comet Rate of Primary Cultured Leydig Cells

Cd^{2+} Concentration (μmol/L)	Normal Cell	Comet Cell	Comet Rate(%)	X^2_{M-H}	*P* Value
0	133	17	11.33		
5	95	55	36.67	26.30	0.001
10	54	96	64.00	88.31	0.001
25	32	118	78.67	136.93	0.001
50	7	143	95.33	211.92	0.001
100	0	150	100.00	238.13	0.001

Note. The number of samples is 150, X^2_{M-H} is R×2 Mantel-Haenszel Chi-Square, *P* value is compared to the 0 μmol/L group.

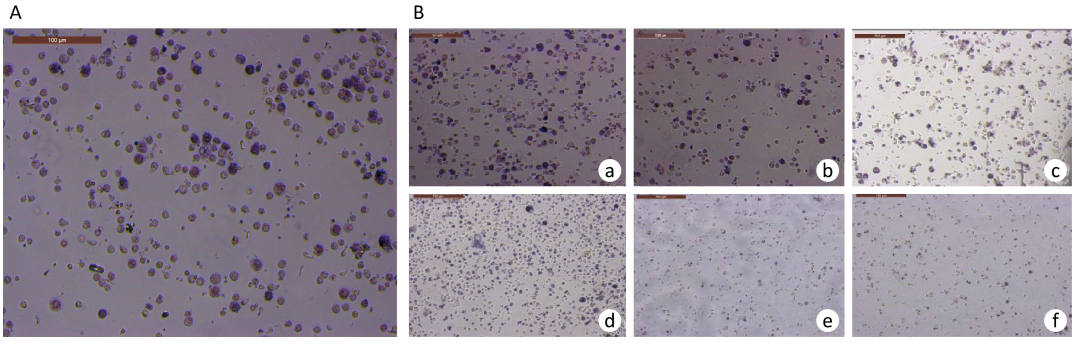

Figure 1. A. Rat Leydig cells after Wiebe staining. B**.** 3β-HSD activity of Leydig cells in primary cultures. a: control; b: 5 μmol/L Cd^{2+} Group; c: 10 μmol/L Cd^{2+} Group; d: 25 μmol/L Cd^{2+} Group; e: 50 μmol/L Cd^{2+}Group; f: 100 μmol/L Cd^{2+}Group.

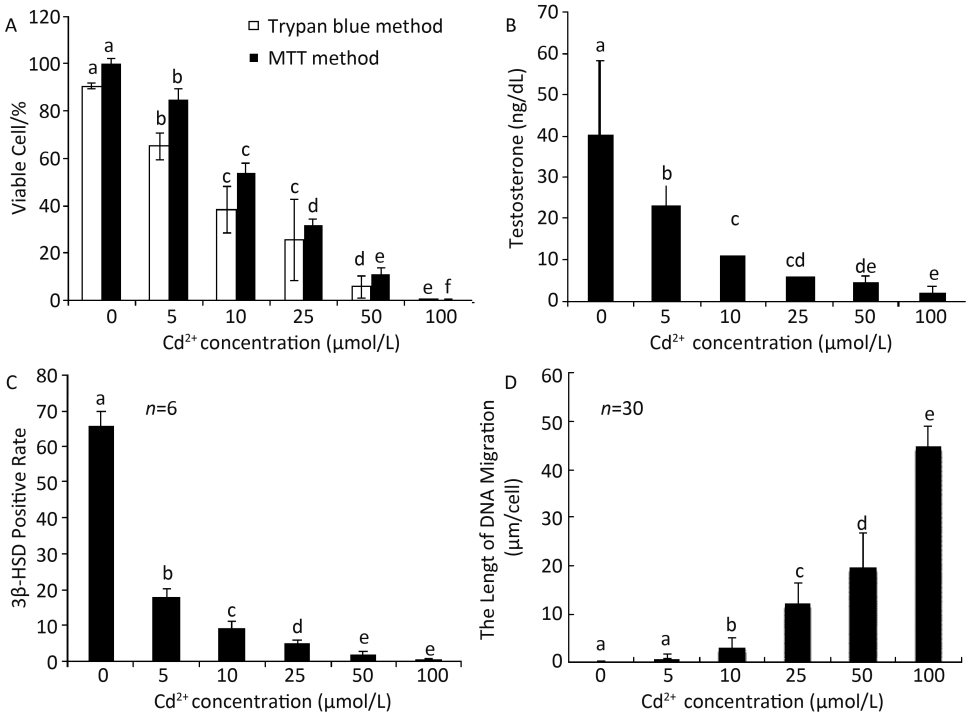

Figure 2. A. Effect of Cd exposure on the viability of Leydig cells in primary culture. B. Effect of Cd on testosterone secretion of Leydig cell in primary cultures. C. Effect of Cd on the 3β-HSD activity of Leydig cells in primary cultures. D. Migration distance of DNA from Leydig cells exposed to cadmium in primary culture. One-way ANOVA test between groups, the same small letter in the same array of the figures denote the non-significant difference (P>0.05), otherwise denote significant (P<0.05).

Testosterone is a key hormone that affects the reproductive function of male animals. The mechanism of testosterone synthesis is a complex process; the hormone is mainly synthesized by Leydig cells and its synthesis depends on the pulsatile secretion of LH by the pituitary gland into the peripheral circulation[9]. 3β-HSD is crucial to testosterone synthesis and is collectively referred to as a steroidogenic enzyme along with P450scc, 17β-HSD, and P450c17a. Reports regarding the influence of Cd on mammalian testosterone synthesis are rare. However, the potential extent of DNA and mechanical damage caused by Cd may affect the expression and catalytic reactions of steroidogenic enzymes.

Cadmium-induced DNA damage occurs in various cells, such as Sertoli cells[10], and liver cells[11]. Normal cells can repair DNA damage and prevent the

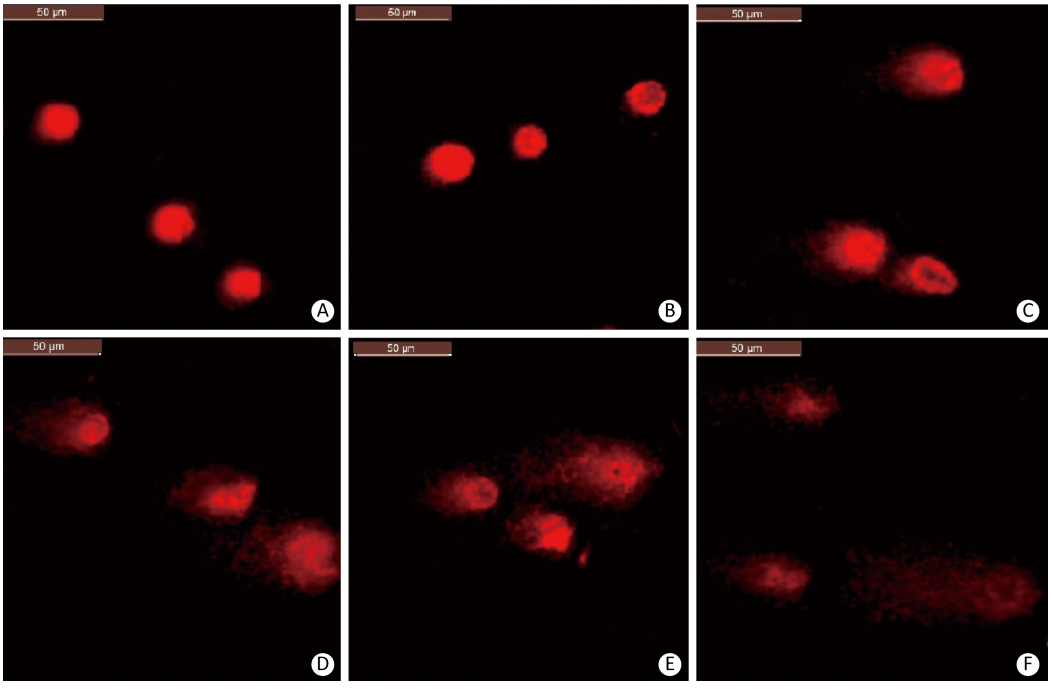

Figure 3. DNA damage of Leydig cells in primary cultures. A: control; B: 5 μmol/L Cd^{2+}Group; C: 10 μmol/L Cd^{2+}Group; D: 25 μmol/L Cd^{2+}Group; E: 50 μmol/L Cd^{2+}Group; F: 100 μmol/L Cd^{2+}Group.

occurrence of mutations; Cd exposure can abrogate the functions of DNA repair genes and potentially increase genomic instability, which may lead to the development of cancer[12]. Singh[13] studied the effects of Cd exposure and its induction of genomic instability in TM3 cells (a mouse testicular Leydig cell line). Their results revealed that the OGG1 and MYH gene expression was decreased within 24 h, which affected the maintenance of DNA methylation, DNMT1 expression, and DNA repair[13]. Yang et al.[14] explored Cd toxicity on primary rat Leydig cells; they concluded that the decreased percentage of normal cells and the increased level of DNA damage in Cd-exposed Leydig cells may account for decreased testosterone secretion.

The results of the current study indicated that Cd was directly toxic to Leydig cells. The dose-response relationship of Cd exposure and cell viability was clearly observed. 3β-HSD is an important enzyme for testosterone synthesis which is only expressed in the Leydig cells of the testis[15]. This enzyme is essential for the biosynthesis of all classes of steroid hormones. This study established a biochemical method to measure the 3β-HSD activity in cultured Leydig cells, such that the effects of Cd exposure on Leydig cell secretion could be directly assessed. Results showed that Leydig cells exposed to Cd had markedly decreased 3β-HSD activity, thereby demonstrating the significant suppression of Leydig cell testosterone synthesis. The observed 3β-HSD activity was consistent with the testosterone concentration in the culture medium. Changes in the testosterone levels of animals exposed to Cd are most probably the direct effect of Cd exposure on Leydig cell secretion. We used the SCGE technique to investigate and demonstrate the DNA damage caused by Cd in Leydig cells. Similar to those of Yang et al.[14], our results suggested that Cd is directly toxic to Leydig cells and induces apoptosis. In addition, the findings of this study established a molecular basis for Cd-induced malformation and carcinogenesis[13]. The mechanism by which Cd causes DNA damage remains inconclusive; DNA repair and oxidative stress are believed to be important mechanisms by which Cd exerts its mutagenic effects[16-17].

In conclusion, we demonstrated that Cd has direct toxic effects on Leydig cells *in vitro*. Cd suppresses Leydig cell testosterone synthesis and secretion, as well as causes significant DNA damage. Thus, we propose that DNA damage, 3β-HSD activity, and testosterone secretion may have reciprocal causes.

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