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·*Original Article*·

Effect of cocaine on germ cell apoptosis in rats at different ages

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Abstract

Aim: To investigate the effect of cocaine on apoptosis and caspase-3 activity in germ cells in male rats at different ages. **Methods:** Cocaine hydrochloride was given (15 mg/kg body weight s.c.) to male Sprague–Dawley rats of 3 weeks (n = 8), 6 weeks (n = 8) and 12 weeks (n = 8) of age, daily for 28 days. The serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), testosterone (T) and estrogen (E_2) were assayed, and the DNA fragmentation of germ cells was determined by gel eletronphoresis. The cell cycle, apoptosis and caspase-3 activity of germ cells were tested by flow cytometry. **Results:** After the 28-day cocaine treatment, testes weight of the 3-week-old rats, the testes and body weights of the 6-week-old rats were decreased significantly compared to those of their corresponding controls ($P < 0.05$). The serum level of T was decreased significantly in the 3-week-old and 6-week-old rats, and the serum level of PRL was also decreased significantly in 12-week-old rats compared to the controls ($P < 0.05$). In all the three cocaine-treated groups, the isolated DNA displayed a clear ladder pattern, especially in the 6-week old rats. The number of apoptosic germ cells increased significantly in 3- and 6-week-old rats treated with cocaine ($P < 0.05$). The caspase-3 activity in all three groups increased significantly compared to the controls ($P < 0.05$), especially in the 6-week-old rats. **Conclusion:** Cocaine exposure for 28 days leads to significant damage to male gonad and apoptosis elevation in testes of rats of different ages, especially in those of 6 weeks of age. The increase in caspase-3 activity might be a key pathway related to the early stage of apoptosis as the mechanism of cocaine-induced germ cell loss. *(Asian J Androl 2006 Sep; 8: 569–575)*

Keywords: cocaine; testis; apoptosis; caspase-3; germ cell

1 Introduction

As a global problem, drug abuse is becoming more and more serious. It is estimated that approximately 29 million American people, 12% of the population in USA, use cocaine [1]. In China, recent epidemiological studies showed that in 2001 around 681 000 people, approximately 0.054% of the Chinese population, were drug abusers, of which 550 000 were male (approximately 80% of the total). Drug abuse is widespread among teenagers around the world and the age of drug abusers is becoming younger. In predominantly white communities in middle class USA, 15% of teenagers (mean age 16.5 years), have used cocaine or "crack" 10 to 50 times

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and 18% (heavy users) have used the drug more than 50 times [2]. In Ontario and British Columbia, nearly 2% of students aged 13–18 years have used crack [3]. In China, drug abusers aged from 17 to 35 years make up 530 000 of drug users, 70–80% of the total. The effects of chronic recreational drug use on the male reproductive system have become a focus of increasing interest in recent years. Cocaine use in humans is believed to lower the sperm count, reduce sperm motility and increase the number of abnormal forms of sperm [3]. In peripubertal male rats, long-term cocaine exposure for more than 100 days can adversely affect spermatogenesis, which, in turn, affects fertility [3]. Cocaine exposure can lead to significant apoptosis in adolescent testes. However, data regarding the effect of cocaine on apoptosis in testes of rats at different ages (impuberal, adolescent and aged rats) are still not available, and the intracellular pathways, such as the caspase family, to germ cell apoptosis have not been fully marked. In the present study, the profiles of germ cell apoptosis induced by cocaine and caspase-3 activity in rat testes at different ages in this process were evaluated by DNA ladder assay and flow cytometry.

2 Materials and methods

2.1 Animal models

In the present study, 3-week-old (n = 16), 6-week-old (n = 16) and 12-week-old (n = 16) male Sprague–Dawley rats (obtained from the Experimental Animal Center of Zhejiang Provincial Medical Institute) were maintained in accordance with National Institutes of Health guidelines [3]. They were divided into three treated groups according to age (3-, 6- and 12-week groups, with 16 rats in each group) with their corresponding controls. All the rats in the treated groups received injections of cocaine (15 mg/kg body weight s.c.; Qinghai Pharmaceutical Factory Co. Ltd, Qinghai, China) dissolved in 0.2 mL distilled water daily, corresponding to an average single dose of a heavy cocaine user [4]. Each rat was weighed every 3 days, and the cocaine hydrochloride dose was adjusted according to the weight. The control groups received 0.2 mL of normal saline daily. The treatment was continued for 28 days. At the end of the 28-day daily exposure, the animals were killed in a CO_2 chamber and their testes were removed, separated, rapidly weighed and frozen in liquid nitrogen, and then stored at –80ºC for DNA isolation and flow cytometry analysis. Blood was collected simultaneously for determination of follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), testosterone (T) and estrogen (E_2) levels.

2.2 Hormone levels measurement

Immunoreactive FSH and LH levels were measured by time-resolved immunofluorometric sandwich assays (DPC, Tianjin, China). FSH and LH immunoreactivity were expressed in terms of the Second International Reference Preparation of Pituitary FSH (code No. 78/549) and the Second International Standard for Pituitary LH (code No. 80/552). The sensitivity of immunofluorometric assay was 0.05 mIU/mL for both gonadotropins, and the intra-assay and inter-assay coefficients of variation (CV) were 4.5% and 5.3% for FSH and 4.9% and 8.5% for LH, respectively. The cross-reactivity of LH in the FSH assay was 0.09% and that of FSH in the LH assay was 0.02%. Serum levels of T, E_2 and PRL were assessed by radioimmunometric assay (RIA) using a coat-account RIA (DPC, Tianjin, China) in accordance with the manufacturer's instructions. The detection limits of T, E_2 and PRL assays were 0.37 ng/mL, 0.04 ng/mL and 1 ng/mL, respectively. The intra-assay and inter-assay CV were 8% and 11% for the T assay, 4.3% and 5.0% for the E_2 assay, and 6% and 10% for PRL assay, respectively. All samples were run in duplicate and assayed by the same investigator, who was blind to the experimental situation.

2.3 DNA ladders

For analysis of DNA fragmentation by agarose gels electorphoresis, the total DNA was isolated from testicular cells. The testes of different groups were decapsulated, and then tissues were homogenized in a lysis buffer (10 mmol/L Tris-HCl, 1 mmol/L ethylenediaminetetraacetic acid [EDTA], pH 7.4). The supernatant was digested with proteinase K (300 μg/mL) at 54ºC for 4 h, and then at 37ºC overnight. DNA was extracted with phenol and then with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1 by volume). The extracted DNA was precipitated overnight in –21ºC ethanol and centrifuged at 10 000 × g for 30 minutes, and the pellet was resuspended in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.5). Subsequently, the DNA was digested with DNase-free RNase (50 μg/mL) at 37ºC for 60 minutes, and the DNA samples, 190 μg/mL per lane, were electrophoretically separated on 1.08% agarose gel. Ethidium bromide was added to the electrophoresis buffer at a final concen-

tration of 0.8 μg/mL [5]. DNA was visualized under an ultraviolet (320 nm) transil-luminalor and the gels were photographed with the Quantity One 4.1.1 Image and Analysis System (Beckman Coulter, Fullerton, CA, USA). The DNA size marker used was a 100 bp Ladder (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.4 Assessment of apoptosis by flow cytometry

For the quantitative analysis of apoptosis in germ cells, a method [6] was used to discriminate the small-sized apoptotic cells from debris using cycle test plus DNA reagent kit (BD Pharmingen, San Diego, CA, USA). The cells were fixed with 70% ethanol, treated with RNase, stained with propidium iodide and analyzed with a flow cytometer (EPICS-XL, Beckman Coulter, Fullerton, CA, USA). The percentage of apoptotic cells in the total cell population was obtained by dividing particle counts in the sub-G_1 apoptotic population by the total counts in the sub-G_1, G_0/G_1, S and G_2/M phase populations and was calculated using Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

2.5 Caspase-3 activity analysis

Caspase-3 activity in cell extracts was measured using an active caspase-3 apotosis kit (BD Pharmingen, San Diego, CA, USA). The cells were collected and lysed in an ice-cold lysis buffer containing 50 mmol/L N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) at pH 7.4, 1 mmol/L dithiothreitol (DTT), 0.1 mmol/L EDTA, 0.1% 3-[(3-cholamidylpropyl)dimethylammonio]-1-propane sulfonate (CHAPS), and 0.1% nonylphenol 40. The cell lysate was centrifuged at 10 000 × *g* for 5 min at 4ºC and the supernatant fraction was used to quantitate caspase-3 activity. Aliquots of 20 μg of cell lysate protein were incubated for 1 h at 37ºC in assay buffer containing 50 mmol/L HEPES at pH 7.4, 100 mmol/L NaCl, 10 mmol/L DTT, 1 mmol/L EDTA, 0.1% CHAPS, 10% glycerol and 0.2 mmol/L Ac-Asp-Glu-Val-Asp-p-nitroaniline substrate. The amount of p-nitroaniline released by caspase-3 activity was quantitated by measuring the optical density at 405 nm. Caspase-3 activity was expressed as pmol p-nitroaniline released per minute per μg cellular protein [7].

2.6 Statistical analysis

Routine statistical data analysis was done using SPSS 10.0 (SPSS, Chicago, IL, USA). All data were expressed as mean ± SD. Statistical analysis was performed using the paired *t*-test. All statistical tests were two-tailed and $P < 0.05$ was used for tests of significance.

3 Results

3.1 Body and testes weights changes in rats at different ages induced by cocaine

After the 28-day treatment, there were no significant differences in body and testes weights in the 12-week-old rats ($P > 0.05$). However, the testes weight of the treated rats reduced significantly in the 3-week group ($P < 0.05$), and both testes and body weights of the treated rats decreased significantly in the 6-week group ($P < 0.05$), when compared with those of the corresponding controls (Table 1).

3.2 The hormone levels of different age rats exposed to cocaine

Table 1. Effects of cocaine on body and testes weights of rats at different ages. The date are expressed as mean ± SD. $^bP < 0.05$, compared with corresponding controls.

Groups	Body weight (g)	Testes weight (g)
3-week (*n* = 16)		
Control group (*n* = 8)	96.38 ± 2.16	0.35 ± 0.06
Cocaine-treated group (*n* = 8)	94.35 ± 2.71	0.25 ± 0.08^b
6-week (*n* = 16)		
Control group (*n* = 8)	221.36 ± 3.77	1.19 ± 0.16
Cocaine-treated group (*n* = 8)	197.96 ± 2.00^b	0.98 ± 0.12^b
12-week (*n* = 16)		
Control group (*n* = 8)	327.68 ± 5.18	1.59 ± 0.12
Cocaine-treated group (*n* = 8)	318.63 ± 3.96	1.54 ± 0.09

In the 3-week-old rats, the T level of rats treated with cocaine was significantly lower, but the E_2 level was significantly higher, than those of the control ($P < 0.05$). In 6-week-old rats, the T and LH levels of rats treated with cocaine were significantly lower than those of the control ($P < 0.01$ and $P < 0.05$, respectively), whereas the FSH level in this group was significantly higher than that of the control ($P < 0.05$). In the 12-week-old rats, only the level of PRL was significantly lower than that of the control ($P < 0.05$) (Table 2).

3.3 DNA fragmentation in cocaine-exposed testes

The occurrence of apoptosis was defined by gel-fractionation analysis of DNA fragmentation. In germ cells of the controls, no DNA fragmentation was found, which was consistent with the low incidence of apoptotic cells, whereas DNA isolated from the testes of cocaine-treated rats displayed a clear ladder pattern when separated by 1.08% agarose gel electrophoresis. DNA fragmentation was increased in size by approximately 200 bp in samples of cocaine-injected rats, especially in those of 6-week and 12-week groups, but it was not detected in the corresponding controls (Figure 1).

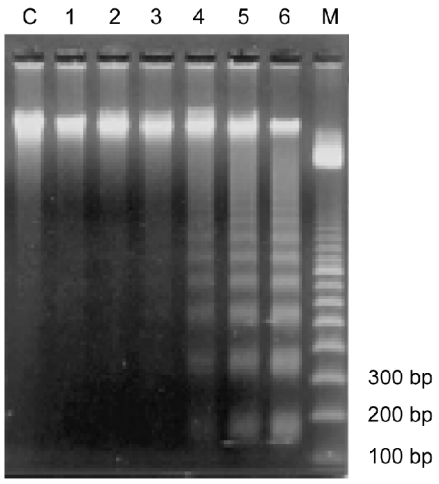

Figure1. DNA fragmentation from testes of cocaine-treated rats and the controls at different ages. Lanes 1 to 3 correspond to low molecular weight DNA fraction isolated from testes of the controls in 3-, 6- and 12-week groups, respectively. Lanes 4 to 6 correspond to low molecular weight DNA fraction isolated from testes of the cocaine-treated rats in 3-, 6- and 12-week groups, respectively. C, Control sample incubated with medium only; M, 100 bp ladder DNA marker.

3.4 Measurement of germ cells apoptosis in testes of cocaine-treated rats

To confirm the induction of apoptosis after cocaine treatment, cell cycle distribution was measured by flow cytometry analysis. As shown in Figure 2, cocaine exposure altered cell cycle distribution according to the reduction of S and G_2/M phase, and the population of sub-G_1 phase increased as evidence of apoptosis in the three groups after the 28-day cocaine treatment, especially for the 6-week group (Figure 2, Table 3).

3.5 Caspase activity assay

To investigate whether caspase-3 is involved in the cocaine-induced apoptosis, we measured its activity in rat germ cells after cocaine treatment. The results demonstrated that the activity of caspase-3 remarkably elevated in rats of these three groups after the 28-day cocaine

Table 2. Changes in hormone levels of rats of different ages after cocaine treament. The data are expressed as mean ± SD. $^bP < 0.05$, $^cP < 0.01$, compared with the corresponding controls. T, testosterone; E_2, estrogen; LH, luteinizing hormone; FSH, follicle stimulating hormone; PRL, prolactin.

Groups	T (ng/mL)	E_2 (pg/mL)	LH (mIU/mL)	FSH (mIU/mL)	PRL (ng/mL)
3-week (n = 16)					
Control group (n = 8)	0.747 ± 0.128	30.028 ± 12.887	2.657 ± 0.607	3.281 ± 1.635	2.625 ± 1.172
Cocaine-treated group (n = 8)	0.452 ± 0.226^b	47.474 ± 7.355^b	2.745 ± 0.594	2.704 ± 1.474	1.729 ± 0.451
6-week (n = 16)					
Control group (n = 8)	2.989 ± 0.623	23.954 ± 4.741	2.639 ± 0.334	2.217 ± 0.807	3.935 ± 0.727
Cocaine-treated group (n = 8)	1.767 ± 0.593^c	25.009 ± 9.737	2.280 ± 0.191^b	3.046 ± 0.781^b	4.077 ± 0.601
12-week (n = 16)					
Control group (n = 8)	4.384 ± 0.924	25.744 ± 6.807	2.761 ± 0.626	2.053 ± 0.879	6.469 ± 1.542
Cocaine-treated group (n = 8)	3.689 ± 0.706	20.508 ± 6.386	2.493 ± 0.281	1.966 ± 0.880	3.649 ± 0.962^c

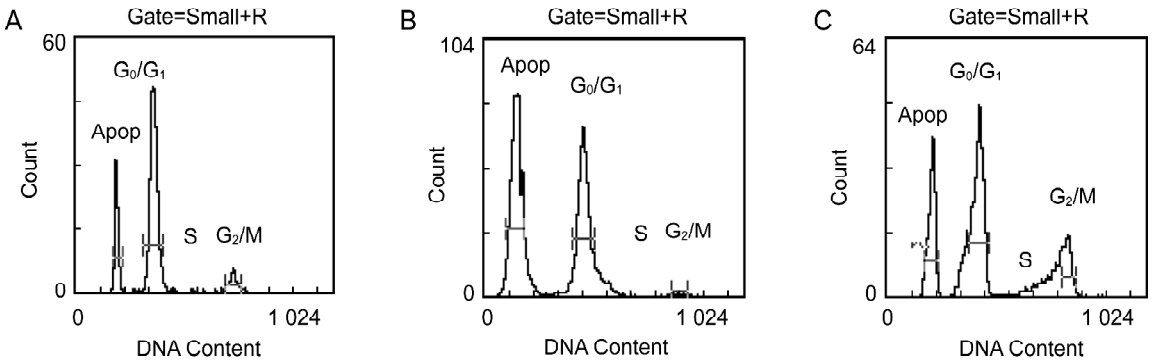

Figure 2. Cell cycle distribution in cocaine-exposed testes. (A): 3-week-old rats. (B): 6-week-old rats. (C):12-week-old rats. Histograms of cells in apoptosis, G_0/G_1, S and G_2/M phase are shown. Apop, apoptosis.

Table 3. Population of sub-G_1 phase and caspase-3 activity in rats at different ages after cocaine treatment. The data are expressed as mean ± SD. $^bP < 0.05$, $^cP < 0.01$, compared with corresponding controls.

Groups	Population of sub-G_1 phase	Caspase-3 activity
3-week (n = 16)		
Control group (n = 8)	25.463 ± 2.353	32.600 ± 4.543
Cocaine-treated group (n = 8)	35.025 ± 2.324^b	40.000 ± 7.456^b
6-week (n = 16)		
Control group (n = 8)	41.086 ± 2.113	66.563 ± 3.023
Cocaine-treated group (n = 8)	50.975 ± 5.305^c	83.988 ± 2.982^c
12-week (n = 16)		
Control group (n = 8)	49.913 ± 4.255	72.563 ± 3.379
Cocaine-treated group (n = 8)	51.650 ± 3.854	78.288 ± 4.733^b

treatment, especially in the 6-week-old rats ($P < 0.01$) (Figure 3, Table 3). In further analysis, the caspase-3 activity was negatively related with the T level in the three cocaine-treated groups ($P < 0.05$) (data not shown).

4 Discussion

In the last decade, considerable data have been accumulated to suggest that recreational drug use can cause damage to the testis. Recent work has shown that chronic administration of cocaine to male rats can adversely affect fertility and spermatogenesis by producing extensive morphological change in the testes, leading to the reduction in sperm production [3]. Research on binding sites of cocaine in tissues shows that the level of cocaine binding to testis cell is lower than that to brain cell, but pronouncedly higher than that to liver, heart and placenta cell [8-11]. George *et al*. [3] demonstrate that after 100 days of treatment, rats receiving daily cocaine treatment have a pregnancy rate of only 33% *vs*. 86% of the controls. Their findings confirm that chronic administration of cocaine to peripubertal male rats has a deleterious effect on spermatogenesis and fertility. However, research on the effect of cocaine exposure on the development and function of testes, and hormone levels at different ages has not been reported so far. In the present study, after the 28-day cocaine treatment, the testes weight of the daily cocaine-treated rats decreased in the 3-week group, and both the testes and body weights of the daily cocaine-treated rats decreased significantly in 6-week-old rats when compared with the controls, respectively ($P < 0.05$). The T level of cocaine-exposed 3-week-old rats was lower, but the E_2 level was higher than that of the control ($P < 0.05$). In 6-week-old rats, the T and LH levels of cocaine-exposed rats were significantly lower than those of the control ($P < 0.01$),

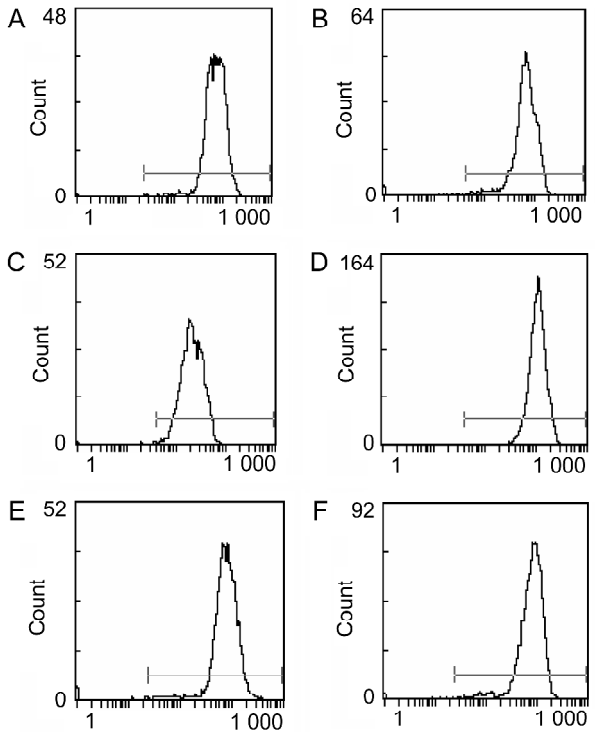

Figure 3. Representative analysis of caspase-3 activity in rat germ cells by flow cytometry analysis. (A): 3-week-old rats treated with saline for 28 days. (B): 3-week-old testes treated with cocaine for 28 days. (C): 6-week-old testes treated with saline for 28 days. (D): 6-week-old testes treated with cocaine for 28 days. (E): 12-week-old testes treated with saline for 28 days. (F): 12-week-old testes treated with cocaine for 28 days. The caspase-3 activity remarkably elevated in all three groups after the 28-day cocaine treatment, especially in the 6-week group ($P < 0.01$).

whereas the FSH level was significantly higher than that of the control ($P < 0.05$). In 12-week-old rats, only the PRL level was significantly lower than that of the control ($P < 0.05$). These data suggest that the 28-day cocaine administration can have a deleterious effect on the function of gonad and reproductive endocrinology in male rats of different ages, which is very serious for peripubertal male rats.

In the study of Li *et al.* [12], a statistically significant increase in germ cell apoptosis was identified as early as 15 days following cocaine injection and persisted up to 90 days, which was in agreement with the histopathology of cocaine-induced testicular atrophy, suggesting that chronic cocaine administration leading to germ cell death in rat testes might be, in part, a result of increased apoptosis. However, the characteristics of cocaine-induced germ cell apoptosis in rats of different ages are yet unknown. Apoptosis has been characterized biochemically by the cleavage of genomic DNA into nucleosomal fragments of 180 bp or their multiples. These fragments are readily detected as a DNA ladder by agarose gel electrophoresis and, in fact, such a ladder pattern has been regarded as the most characteristic hallmark of apoptosis [7]. Our results demonstrate a clear DNA ladder pattern followed cocaine injection in three groups of rats at different ages, suggesting that cocaine exposure might not only lead to pronouncedly significant apoptosis in peripubertal male rat testes, but also lead to apoptosis in young and aged rat testes. Furthermore, the cell cycle distribution measured by flow cytometry analysis in the present study shows that cocaine exposure can alter cell cycle distribution according to the reduction of S and G_2/M phase, and the population of sub-G_1 phase increased as evidence of apoptosis in the three groups after the 28-day cocaine treatment, especially in the 6-week group. The damage of germ cells induced by cocaine might be a result of the increase of apoptosis.

Cocaine is rapidly metabolized by two distinct pathways (hydrolytic and oxidative reaction), with less than 5% excreted unchanged in the urine [13]. Apoptosis caused by cocaine exposure can be a result of cocaine itself and/or its metabolites. Some important genes or molecules in apoptosis, such as fas/fas ligand, bcl-2/bax and p53, are present in the testis [14]. However, the intracellular pathways, such as the caspase family, to germ cell apoptosis have not been fully marked. During the past few years, rapid advances in the molecular understanding of apoptotic mechanism have identified numerous pathways for the cell death. The execution phase involves a series of morphological and biochemical changes that appear to result from the action of cysteine-dependent aspartate-directed proteases called caspases [15]. The caspase family, containing at least 14 kinds of human cysteine proteases, appears to play a critical role in initiating and sustaining biochemical events that result in apoptotic cell death. They are synthesized as inactive proenzymes comprising an N-terminal peptide (pro-domain) together with large and small subunits. Activation of caspases during apoptosis results in the cleavage of an enzymatically active heterotetramer with activity. Caspases trigger a cascade of proteolytic cleavage events and are considered central players in all apoptotic events in mammals [16]. Of these cysteine proteases, caspase-3 is believed to be one of the most

commonly involved in the execution of apoptosis in various cell types [15] and a key protease activated during the early stages of apoptosis [16]. Our study shows that the activity of caspase-3 can remarkably elevate in rats of 3 and 6 weeks of age after the 28-day cocaine treatment, especially in 6-week-old rats, suggesting that caspase-3 might play an important role during the early stages of apoptosis as the mechanism of cocaine-induced germ cell loss.

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