Effect of immobilization stress on testicular germ cell apoptosis in rats

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The influence of immobilization stress on testicular germ cell apoptosis was investigated in rats. A transient increase in serum corticosterone and a transient decrease in serum testosterone were observed during each period of immobilization stress. Twenty-four hours after the last immobilization session, the testicular weight and serum concentrations of corticosterone and testosterone were the same between the immobilization stress and control groups. However, the percentages of apoptotic tubules and apoptotic cells in the stress group were significantly higher than those in controls \((P < 0.001)\). These facts suggest that immobilization stress can enhance testicular germ cell apoptosis in rats.

Key words: apoptosis/corticosterone/germ cell/rat/testosterone

Introduction

The spontaneous death of testicular germ cells appears to be a constant feature of normal spermatogenesis in a variety of mammalian species (Huckins, 1978; Allan et al., 1992; Kerr, 1992). Testicular germ cell death is found most commonly in the spermatogonia, but it also occurs spontaneously throughout spermatogenesis (Huckins and Oakberg, 1978). Recent studies have provided evidence that the degeneration of testicular germ cells involves apoptosis (Allan et al., 1992; Tapanainen et al., 1993). In addition, germ cell apoptosis has been experimentally demonstrated in response to androgen withdrawal (Tapanainen et al., 1993) and hyperthermia (Shikone et al., 1994).

Reproductive function in male primates or animals is suppressed by psychogenic or somatic stress (Sapolsky, 1985; Orr and Mann, 1992). Also, in rats, immobilization or restraint stress usually lowers plasma testosterone concentrations (Charpenet et al., 1982). To our knowledge, however, there is no literature dealing with the relationship between immobilization stress and testicular germ cell apoptosis.

Materials and methods

Animals

The following experiments were conducted in accordance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals. In addition, all regulations on experimental animal studies recommended by Japanese Law were adopted. Young adult male rats (250–300 g) of the Sprague-Dawley strain were purchased from Clea Japan Inc. (Tokyo, Japan). The animals were housed 2–3 per cage in hanging wire mesh cages under controlled lighting conditions (14 h light beginning at 0600 and 10 h dark) at a temperature of 20–24°C. They were handled daily for 1 week prior to the experiments.

Immobilization protocol and blood sampling

The animals were divided into two groups. In the immobilization stress group, 10 rats were subjected to repeated immobilization stress for 2 h by placement in a 6 cm diameter metal cylinder. The immobilization session began at 0600 h, and it was repeated each day for 7 days. In the control group, the 10 rats were left undisturbed.

In the immobilization stress group, blood samples were collected before and 30, 60 and 90 min after the start of the immobilization session, as well as 60 min after the end of the session using the tail clip method. The animals were killed 24 h after the last immobilization session by decapitation and their trunk blood was collected between 0800 and 0900 h. Each sample was centrifuged, and the serum was stored at –80°C until measurement. Body weight was recorded, and the bilateral testes were removed and weighed.

Hormone analysis

The serum corticosterone concentration was measured by radioimmunoassay using an antiserum raised in rabbits against corticosterone-3 coupled to bovine serum albumin and using 3H-corticosterone as a radiolabelled ligand (Biogenesis Ltd, London, UK). The within- and between-assay coefficients of variation were 4.7 and 6.8% respectively. The detection limit was 2 pg/ml. The serum concentration of testosterone was determined by a solid tube radioimmunoassay method. A radioimmunoassay kit (Diagnostic Product Corporation, Los Angeles, CA, USA) was used for the measurement of testosterone. The within- and between-assay coefficients of variation were 7.0 and 7.9% respectively. The detection limit was 0.1 ng/ml. All samples were assayed together in duplicate.

DNA extraction

The tissues were decapsulated and minced. Low molecular weight DNA fragments were isolated by lysis of the minced tissue at 37°C for 30 min in 10 volumes of buffer (10 mmol/l Tris, HCl, pH 8.0, 0.1 mol/l ethylemediamine-tetra-acetic acid [EDTA], pH 8.0, 0.5 sodium dodecyl sulphate [SDS] containing RNase A and proteinase K at the final concentrations of 40 µg/ml and 100 µg/ml respectively). NaCl was added to a final concentration of 1 mol/l, and the solution was incubated at 4°C overnight. A 30 min centrifugation at 10,000 g followed to pellet the high molecular-weight masses. The supernatant
Stress and testicular germ cell apoptosis

Figure 1. Serum corticosterone concentrations before, during, and after each immobilization session. Vertical bar represents the standard deviation. *** $P < 0.001$ compared to pre-immobilization levels.

Figure 2. Serum testosterone concentrations before, during and after each immobilization session. The vertical bar represents the standard deviation. ** $P < 0.01$ compared to pre-immobilization levels.

was extracted with two volumes of phenol:chloroform:isoamyl alcohol (25:24:1) mix and precipitated with two volumes of ethanol for at least 2 h at −20°C. After centrifugation at 10 000 g for 30 min, the DNA pellet was resuspended in buffer (10 mmol/l Tris, HCl, pH 7.5, 1 mmol/l EDTA) and electrophoresed on a 1.5% agarose gel.

**DNA end labelling of tissue sections (TUNEL method)**

Tissue sections were excised from the testes, embedded in Tissue-Tek® (Miles Inc., Elkhart, IN, USA) and stored at −70°C. Frozen sections, 5 μm thick, were mounted on silane-coated glass slides (Dako Japan, Tokyo, Japan) and fixed for 15–30 min at room temperature in freshly prepared 4% paraformaldehyde buffered with 0.1 mol/l sodium phosphate (pH 7.4). Endogenous peroxidase was inactivated by 0.3% H$_2$O$_2$ for 15–30 min at room temperature. To permeabilize the sections, they were incubated with a permeabilization buffer for 5 min at 4°C. In-situ end-labelling was performed using an in-situ apoptosis detection kit (Takara Biomedicals, Tokyo, Japan), comprised of non-radioactive fluorescein-dideoxyuridine triphosphate (dUTP). The sections were incubated with TdT and fluorescein-dUTP at 37°C for 60–90 min in a humidified chamber, and 3′-OH ends of the DNA fragments were tailed with fluorescein. The sections were then washed three times in PBS. After being incubated with anti-FITC horseradish peroxidase conjugate for 30 min at 37°C, the slides were washed three times in PBS, developed with 0.05% diaminobenzidine, and stained for 10–15 min at room temperature. The specimens were then washed three times in distilled water, counterstained by Mayer’s haematoxylin solution for 5–10 min, dehydrated and then mounted. For the evaluation of apoptosis, microscopic fields were selected at random. The percentage of the apoptotic germ cells was determined by counting a total of 1000 germ cells from
Corticosterone (µg/ml) 1.1
Testosterone (ng/ml) 6

The immobilization stress testes were the same for both the immobilization stress and immobilization value (Figure 2).

Each start of the immobilization session. At 60 min after each concentration also showed a significant reduction 90 min after the start of each immobilization. At 90 min after the start of immobilization stress, the serum testosterone concentration returned to the pre-immobilization value. The serum testosterone concentration was found at each measurement until 60 min after the start of the each immobilization. As shown in Figure 1, a drastic elevation of serum corticosterone and testosterone in control and immobilization stress rats

Table I. Testicular weight and serum concentrations of corticosterone and testosterone in control and immobilization stress rats

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control (10)</th>
<th>Immobilization stress (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testicular weight (g)</td>
<td>2.9 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>Corticosterone (µg/100ml)</td>
<td>2.3 ± 0.3</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>1.1 ± 0.2</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
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Each value represents mean ± SD. ( ) represents number of animals.

The results were analysed for statistical significance using Student’s unpaired t-test. Differences were considered to be statistically significant if P < 0.05.

Results
As shown in Figure 1, a drastic elevation of serum corticosterone was found at each measurement until 60 min after the start of the each immobilization. At 90 min after the start of the immobilization session, the corticosterone concentrations began to reduce, and 60 min after the end of the immobilization session, the serum concentrations of corticosterone returned to the pre-immobilization value. The serum testosterone concentrations also showed a significant reduction 90 min after the each start of the immobilization session. At 60 min after each immobilization session, however, they returned to the pre-immobilization value (Figure 2).

Twenty four hours after the last stress session, the testicular weight and serum concentrations of corticosterone and testosterone were the same for both the immobilization stress and the control groups (Table I). The immobilization stress testes demonstrated DNA fragmentation (Figure 3), but no DNA fragmentation was observed in the control testes (Figure 3). The control testes showed normal architecture of seminiferous epithelium without signs of apoptosis (Figure 4A), but the immobilization stress testes stained positive for apoptotic DNA (Figure 4B). Positive staining was observed most frequently in the spermatogonia and primary spermatocytes. The percentages of apoptotic tubules and apoptotic cells in the immobilization stress group were significantly higher than those in controls (P < 0.001)(Figure 5).

Discussion
Chronic immobilization stress usually lowers serum testosterone concentrations (Sapolsky, 1985). Immobilization stress also provokes an elevation of serum corticosterone concentrations which causes the decline in testosterone concentrations (Srivastava et al., 1993). In the present study, similar hormonal changes were observed during immobilization stress. It is postulated that during stress, increased corticosteroids suppress gonadotrophins (Bambino and Hsueh, 1981; Ringstrom and Schwartz, 1987) and directly suppress testicular function (Sapolsky, 1985). In addition, stress-mediated increases in corticotrophin-releasing factor (CRF) result in a reduced secretion of the gonadotrophin-releasing hormone (GnRH)(Gindoff and Ferin, 1987). It has been suggested that CRF acts directly in suppressing GnRH or indirectly by increasing opioid release (Gindoff and Ferin, 1987; Kostic et al., 1997). In the present study, however, corticosterone concentration returned to the control value 90 min after the start of immobilization stress and testosterone concentration returned to the control value 60 min after the end of each immobilization session. In addition to this, 24 h after the last stress session, both corticosterone and testosterone concentrations returned to the pre-immobilization values. According to Suarez, 2 h of immobilization stress for 12 consecutive days did not enhance basal serum corticosterone concentrations (Suarez et al., 1996). Therefore, 2 h of immobilization stress for 7 consecutive days was not enough to increase basal testosterone concentrations and to suppress the basal testosterone concentration.

Severe stress of a chronic or intermittent duration can have detrimental effects on reproductive processes in animals. Exposure of the testis to the relatively elevated temperature of the abdomen is known to cause the loss of germ cells (Mengal et al., 1981). Also, experimentally induced cryptorchidism shows a clear temporal relationship between the onset of DNA fragmentation by day 6 and germ cell loss on day 7 (Yin et al., 1998). Thus, it seems that the testicular germ-cell loss observed with exposure to abdominal heat stress occurs by apoptosis. It was reported (Suarez et al., 1996) that 2 h of immobilization stress for 24 consecutive days results in the modification of sperm function activity. However, the effect of stress on the rate of spermatogenesis has not been reported. The present study showed that 2 h of immobilization stress for 7 consecutive days was sufficient to increase testicular germ cell apoptosis in normal basal concentrations of serum corticosterone and testosterone. However, a transient increase in the serum corticosterone and a decrease in serum testosterone
was observed during each immobilization session. When adult male rats are treated with ethane dimethane sulphonate, an agent which leads to testosterone withdrawal by the selective destruction of Leydig cells, the occurrence of germ cell loss by apoptosis increases remarkably (Troiano et al., 1994). Thus, apoptotic cell death in the seminiferous epithelium is thought to be mediated by androgens. Therefore, a transient reduction of serum testosterone caused by immobilization stress can enhance testicular germ cell apoptosis in rats. In the human testis, however, apoptotic germ cells are present in the spermatogenic epithelium of untreated patients and patients who received short-term anti-androgen treatment (Brinkworth et al., 1997; Woolveridge et al., 1998). Despite the marked fall in androgen support to the spermatogenic epithelium after short-term anti-androgen treatment, the concentrations of the apoptosis-related proteins, such as BCL-2, BCL-XL, BAX, P53 and poly (ADP) ribose polymerase (PARP), do not change. In contrast, there are few or no apoptotic cells in the seminiferous tubules following long-term anti-androgen treatment. In the long-term treated testes, BCL-XL and PARP expression decline, BAX and P53 protein concentrations are unchanged, and BCL-2 is up-regulated (Woolveridge et al., 1998). Thus, observations of apoptosis in the human testis are complicated by the presence of many different cell types, which are maintained in a complex architectural arrangement. Even though BCL-2, BAX and BCL-XL are unchanged, they may play a role at another time point after androgen withdrawal. Further investigations should be performed in order to clarify...
Figure 5. Percentages of apoptotic tubules and apoptotic cells in control and immobilization stress rats. The vertical bar represents the standard deviation. ***P < 0.001 compared to controls.

the involvement and cellular localization of these gene products following androgen withdrawal.

References


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