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# Mild Testicular Hyperthermia Induces Profound Transitional Spermatogenic Suppression Through Increased Germ Cell Apoptosis in Adult Cynomolgus Monkeys (*Macaca fascicularis*)(

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## Abstract

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We have previously demonstrated that mild testicular hyperthermia induces stage-specific and germ cell—specific apoptosis in rat and mouse testes. The objectives of this pilot study were to examine whether mild testicular hyperthermia induces azoospermia and oligozoospermia in nonhuman primates, and to determine whether spermatogenesis suppression was due to acceleration of germ cell apoptosis. Three adult Cynomolgus monkeys (*Macaca fascicularis*) were used in this study. The scrota containing the testes were immersed in a water bath at 43°C for 30 minutes once daily for 6 consecutive days. Semen and blood samples were collected at 2 and 1 weeks before, and 2, 4, 6, 8, 10, and 12 weeks after the first heat treatment. Testicular biopsies were performed before and at 3 and 7 days, and 12 weeks after the first heat exposure. Apoptosis in testicular biopsy was assessed by TUNEL assay, by electron microscopy, and by detection of cleaved Poly(ADP-ribose)polymerase with Western blotting. A transient decrease in serum testosterone levels was observed in 2 monkeys 2 weeks after heat treatment. Serum inhibin B levels declined in all 3 monkeys 2 weeks after testicular hyperthermia and remained at relatively low levels throughout the study in 2 of 3 monkeys. Two of 3 monkeys exhibited azoospermia by 6 or 8 weeks after the first heat treatment; the remaining monkey had marked oligozoospermia (8 x $10^6$/ejaculate, 10.89% of pretreatment levels) 6 weeks after the first heat treatment. Increased germ cell apoptosis in testicular biopsy samples was found at 3 and 7 days after the first heat exposure. Using immunohistochemistry, we observed that the immunoactivity of proapoptotic Bax protein accumulated in heat-induced apoptotic germ cells. Full recovery of spermatogenesis was

noted 12 weeks after the first heat treatment. We conclude that, similar to rodents, mild testicular hyperthermia results in azoospermia and oligozoospermia in monkeys through increased germ cell apoptosis with minimal effect on the hormonal milieu.

Key words: Azoospermia, germ cell death, heat, primate, spermatogenesis

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We have previously demonstrated that a single exposure (43° C for 15 minutes) of rat testis to heat results in selective but reversible damage to the seminiferous epithelium through increased germ cell apoptosis. Heat-induced germ cell apoptosis predominantly occurred at the early (I—IV) and late (XII—XIV) stages. Pachytene spermatocytes and early round spermatids at stages I—IV and pachytene, diplotene, and dividing spermatocytes at stages XII—XIV were most susceptible to heat (Lue et al, 1999). We have further demonstrated that redistribution of the proapoptotic Bax protein from a cytoplasmic to a perinuclear location is an early step in the apoptotic pathway leading to germ cell death triggered by mild testicular hyperthermia (Yamamoto et al, 2000). We have also provided evidence indicating that intratesticular testosterone plays a pivotal role in protecting germ cells at stages VII—VIII against heat-induced cell death (Lue et al, 1999).

In additional studies, we have shown that in rats, a single testicular heat exposure (hit 1) in combination with a well-studied hormonal contraceptive (exogenous testosterone administration; hit 2) rapidly and markedly suppresses spermatogenesis to near azoospermia through increased germ cell apoptosis to a greater extent than either testosterone or heat alone (Lue et al, 2000a). Meiosis and spermiogenesis are the most vulnerable phases to respond to testosterone plus heat treatment. These findings suggest that a combination of hormonal treatment such as testosterone implants and a physical agent such as heat exposure is more effective in suppressing spermatogenesis than either treatment alone. We proposed that a combination of 2 antispermatogenic agents (ie, 2 hits) working at separate stages of the spermatogenic cycle might lead to greater male contraceptive efficacy.

In this pilot study we used a nonhuman primate as a model for studying human spermatogenesis in order to examine the effects and mechanisms of action of mild testicular hyperthermia. Effects of gonadotropin deprivation after gonadotropin—releasing hormone antagonist or exogenous administration of testosterone on spermatogenesis in Cynomolgus monkeys have been previously reported (Weinbauer et al, 1998; Zhengwei et al, 1998; O'Donnell et al, 2001). In contrast to that for hormonal intervention, the effects of testicular hyperthermia on spermatogenesis have been examined in humans but they have been rarely documented in monkeys (Watanabe, 1959; Venkatachalam and Ramanathan, 1962; Kandeel and Swerdloff, 1988; Mieusset and Bujan, 1995; Setchell, 1998). The objectives of this study were to examine whether mild testicular hyperthermia induces azoospermia, oligozoospermia, or both in nonhuman primates; and to determine whether the suppression of spermatogenesis was due to acceleration of germ cell apoptosis.

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### Animals and Experimental Protocol

Three adult male Cynomolgus monkeys (*Macaca fascicularis*) (5-7 kg) housed at the Primate Center at the University of California at Davis were used for this study. Animals were kept in a standard animal facility in a controlled temperature (22° C) and photoperiod (12 L:12 D), with free access to water and monkey chow. Under light sedation with Telazol (5-8 mg/kg), testicular hyperthermia was induced

by immersing the monkey scrota containing the testes into a water bath at 43° C for 30 minutes. After heat treatment, animals were dried, examined for any redness or injury to the scrota, then returned to their cages and allowed to recover from the effect of the anesthesia. Inspection of the scrota immediately after and during subsequent heat exposures showed no evidence of thermal injury to the scrotal skin after this short duration of modest increase in temperature. Animal handling and experimentation were in accordance with the recommendation of the American Veterinary Medical Association and were approved by the California Regional Primate Research Center Animal Care and Use Review Committee.

Local testicular heating was carried out in a water bath at 43° C for 30 minutes once daily for 6 consecutive days. Semen and blood samples were collected 2 and 1 weeks before and 2, 4, 6, 8, 10, and 12 weeks after the first heat treatment. Serum testosterone and inhibin B levels were measured by radioimmunoassay and enzyme-linked immunosorbent assay, respectively. Testicular biopsies were performed at 3 and 7 days, and 12 weeks after the first heat exposure.

### *Semen Analysis*

Males were trained to chair restraint and were electroejaculated with a Grass 6 stimulator equipped with electrocardiogram pad electrodes for direct penile stimulation (Sarason et al, 1991). The volume of each ejaculate including both fluid and coagulum fractions was recorded, and the total sperm number was determined from the fluid fraction using a hematocytometer and expressed as spermatozoa x $10^6$/ejaculate (Tollner et al, 1990). Sperm motility in the fluid fraction was evaluated with computer-assisted sperm analysis (Vandevoort et al, 1994). Sperm head morphology was assessed from seminal smears as described previously (Gago et al, 1999). To avoid artifacts produced during preparation of smears, morphology of sperm flagella was assessed with live sperm adjusted to a concentration of 2-3 x $10^6$/mL. At 200x magnification, 200 sperm chosen at random were manually scored for each category of tail defect. For each category, counts were reported as a percentage of the total number of scored sperm.

### *Blood Collection and Testicular Biopsy*

Blood samples were collected from an arm vein while the animals were briefly restrained, and serum was separated and stored at -20° C for subsequent hormone assays. Open testicular biopsies were performed under aseptic conditions. Testicular tissue from each animal was divided equally into 3 portions. One portion of testicular tissue was immersion-fixed with 5% glutaraldehyde in 0.05 M cacodylate buffer pH 7.4, 1 portion was immersion-fixed in Bouin solution, and the remaining portion was snap-frozen in liquid nitrogen and stored at -70° to -80° C for subsequent analysis of proteins by Western blotting. Testicular biopsies were placed into the respective fixatives overnight and processed for routine paraffin embedding for in situ detection of apoptosis and immunohistochemical studies. Portions of glutaraldehyde-fixed testes were embedded in Epon 812 (Polyscience, Warrington, Pa). Thin sections from selected tissue blocks were cut with an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Hitachi 600 electron microscope (Tokyo, Japan; Sinha Hikim et al, 1997a).

### *Hormone Assays*

Testosterone concentrations in plasma were measured by radioimmunoassay as reported previously (Wang et al, 1993). The lower limit of quantitation was 0.25 ng/mL. The intra-assay and interassay coefficients of variations were 8% and 11%, respectively. Plasma inhibin B levels were measured by enzyme-linked immunosorbent assay (University of California, Davis) as described earlier (Shideler et al, 2001). The minimal detection limit in the assay was 0.02 ng/mL. The intra-assay and interassay coefficients of variation were 6% and 8%, respectively.

### Assessment of Apoptosis

In situ detection of apoptosis was performed in glutaraldehyde-fixed, paraffin-embedded testicular sections by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technique using an ApopTag-peroxidase kit (Intergen Co, Purchase, NY) as described earlier (Sinha Hikim et al, 1997b). The TUNEL assay in testicular tissues from monkeys had been optimized as reported previously (Lue et al, 1998). The method used for germ cell quantitation was similar to that described previously (Sinha Hikim et al, 1998; Lue et al, 2001). In brief, testicular sections were examined with an American Optical Microscope (Scientific Instruments, Buffalo, NY) with a 40x objective and a 10x eyepiece. A square grid fitted within the eyepiece provided a reference area of 62 500 $\mu m^2$. Apoptotic germ cells within the frame of grid were counted.

### Western Blot Analysis

Protein extraction and Western blotting were performed as previously described (Yamamoto et al, 2000). Briefly, 100 µg of protein was resolved on a 7.5% sodium dodecyl sulfate polyacrylamide gel at 160 V in a Mini-Protean II Cell (Bio-Rad Laboratories Inc, Hercules, Calif). Equal loading was examined by running a separate gel in parallel and staining with Coomassie blue. Proteins were transferred to 0.45 µm nitrocellulose membranes in transfer buffer (25 mM Tris base, 190 mM glycine, 20% methanol) at 100 V for 1 hour in the cold. Efficiency of transfer was determined using Ponceau S (Sigma Chemical Company, St Louis, Mo). Membranes were blocked in 5% non-fat dried milk in TTBS (0.9% NaCl, 0.1% Tween-20, 100 mM Tris-HCl, pH 7.5) and then incubated with the primary antibody (1:200 poly(ADP-ribose) polymerase [PARP]; Santa Cruz Biotechnology Inc, Santa Cruz, Calif) for 1 hour at room temperature. Following three 10-minute washes in TTBS, membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit (Amersham Life Science Inc, Arlington Heights, Ill) secondary antibodies at a 1:2000 dilution. For immunodetection, membranes were incubated with SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, Ill) and exposed to Fuji x-ray film (Fuji Medical Systems Inc, Stamford, Conn). To confirm the specificity of the PARP antibody, protein extracts of rat testes treated by mild hyperthermia with or without primary antibody were used as controls.

### Immunohistochemical Analysis

Bouin-fixed, paraffin-embedded testicular sections were deparaffinized, hydrated in a successive series of ethanol, rinsed in distilled water, and then incubated in 2% $H_2O_2$ to quench endogenous peroxidases. Sections were blocked with 5% normal goat serum for 20 minutes to suppress nonspecific binding of immunoglobulin (Ig) G and subsequently incubated with a 1:200 dilution of Bax affinity-purified rabbit polyclonal antibody (Santa Cruz Biotechnology). Immunoreactivity was detected using biotinylated goat anti-rabbit IgG secondary antibody followed by avidin-biotinylated horseradish peroxidase complex visualized with diaminobenzidine tetrahydrochloride according to the manufacturer's instructions (Rabbit United Immunohistochemistry Detection System; Oncogene, Boston, Mass). Slides were counterstained with hematoxylin and reviewed with an Olympus BH-2 light microscope.

## Results



### Semen Analysis

Total sperm counts from each semen sample of the 3 monkeys at different time points are shown in Figure 1. In 2 of 3 monkeys, no sperm was detected in the semen by 6 and 8 weeks after the first heat treatment; the remaining monkey

became oligospermic ($8 \times 10^6$/ejaculate) 6 weeks after the first heat exposure, and the sperm concentration decreased to 10.89% of pretreatment levels (this was used as a control). Total sperm count recovered to pretreatment levels 12 weeks after the first heat treatment. No significant changes in sperm motility and morphology were noted after testicular heat exposure (data not shown).

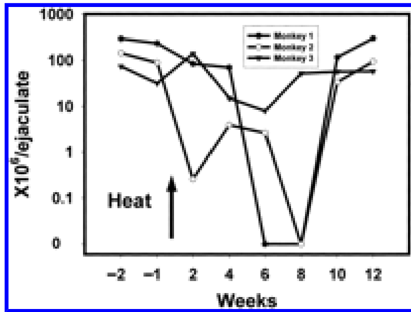


Figure 1. Testicular hyperthermia induces azoospermia in monkeys 1 and 2 and oligozoospermia in monkey 3. Total sperm count recovered to baseline levels 12 weeks after the first heat treatment. Note the sperm concentration is in logarithmic units.

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## *Hormone Levels*

Serum testosterone and inhibin B levels before and after mild testicular hyperthermia are displayed in Figure 2. A transient decrease in serum testosterone levels was noted in 2 of 3 monkeys 2 weeks after heat exposure (Figure 2A). Serum inhibin B levels (Figure 2B) were markedly decreased in all 3 monkeys 2 and 4 weeks after the first heat treatment, and remained suppressed through weeks 4 to 12 in two of the monkeys.

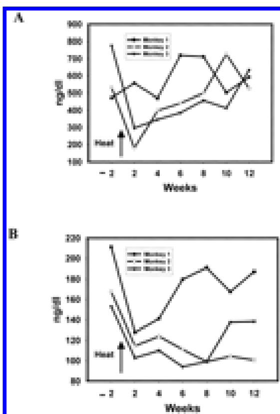


Figure 2. Serum testosterone **(A)** and inhibin B **(B)** levels before and after heat treatment. A transient decrease in serum testosterone levels was noted in 2 of 3 animals 2 weeks after heat treatment, and testosterone levels returned to normal thereafter. A decline in inhibin B levels was present in all monkeys after heat exposure. Serum inhibin B levels remained at relatively low levels in these 2 animals 2 weeks afterward, until at the end of the study. Serum inhibin B levels rebound to normal levels in the remaining monkey.

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## *Heat-Induced Activation of Germ Cell Apoptosis*

Mild testicular hyperthermia once daily for 6 days resulted in a marked but patchy increase in the incidence of germ cell apoptosis, mainly in pachytene spermatocytes and round spermatids, in some but not all seminiferous tubules at both day 3 and day 7 after the first heat treatment (Figure 3).

The quantitative data showed that the incidence of germ cell apoptosis was increased at both day 3 (22-35 apoptotic cells/$10^5 \mu m^2$) and day 7 (33-38 apoptotic cells/$10^5 \mu m^2$) after the first heat exposure compared with pretreatment controls (1-2 apoptotic cells/$10^5 \mu m^2$). By week 12, spermatogenesis completely recovered and the incidence of germ cell apoptosis was similar to pretreatment control levels. Electron microscopic examination further confirmed the incidence of germ cell apoptosis. Germ cells displaying characteristic features of various stages of apoptosis were readily observed in heat-treated testes both at day 3 and day 7 (Figure 4). To substantiate our morphological finding of heat-induced apoptosis, we evaluated the possible changes in PARP in heat-treated testicular tissues. PARP is a constitutively expressed, abundant nuclear protein involved in DNA repair. PARP is cleaved by caspase 3 and results in failure of DNA repair in apoptotic cells (Jimenez et al, 2002). Thus, in this study, the presence of cleaved PARP is used as an additional biological mark for apoptosis. The results from this study showed that PARP was decreased after heating as a result of the presence of cleaved PARP in heat-treated testes (Figure 5).

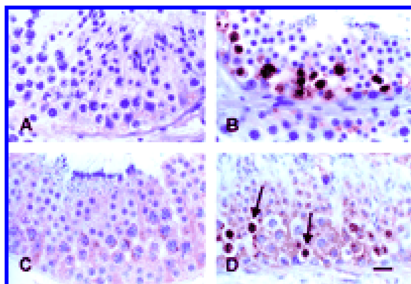


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Figure 3. In situ detection of germ cell apoptosis in monkeys before **(A)** and after heat treatment **(B)**. Cellular localization of apoptosis was characterized by TUNEL assay. Methyl green was used as a counterstain. Many apoptotic germ cells (dark brown in color) were noted in testicular biopsies obtained 3 days after the first heat treatment **(B)** but not from a control animal **(A)**. Bax immunoactivity in the testis before **(C)** and after heat treatment **(D)**. A portion of seminiferous tubule from a normal adult monkey showing that Bax is localized in the cytoplasm of germ cells, Sertoli cells, and a little in Leydig cells **(C)** and from a monkey 3 days after the first heat treatment **(D)** displaying intensive immunoactivity of Bax in apoptotic germ cells (arrow). Magnification, 420x. Scale bar = 25 µm.

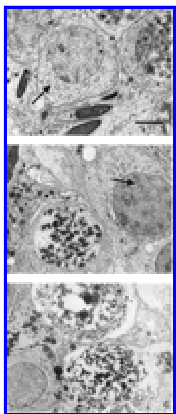

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Figure 4. Electron micrographs of a testicular biopsy from a monkey that had been exposed to short-term (30 minutes) local testicular heating once daily for 6 days showing **(A)** a normal pachytene spermatocyte (arrow) and a pachytene spermatocyte at early stage of apoptosis (*); **(B)** a normal pachytene with synaptonemal complex (arrow) and an apoptotic pachytene spermatocyte with more chromatin condensation; and **(C)** 2 pachytene spermatocytes at late stage of apoptosis with prominent chromatin condensation and fragmentation (*). Biopsies were fixed with 5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanols, and embedded in Epon 812. Magnification, 4000x. Scale bar = 3.75 µm.

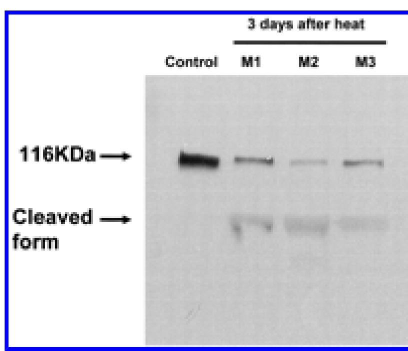


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Figure 5. PARP cleavage in testicular biopsies from a pretreatment control and 3 monkeys 3 days after the first heat treatment are shown by Western blotting. In control testis, the molecular weight of PARP is 116 kd. Note the presence of a cleaved form of PARP 3 days after the first heat treatment.

Changes in the in vivo pattern of Bax expression after heat-induced germ cell apoptosis were examined by immunohistochemistry (Figure 3). In normal monkey testis, Bax protein was localized in the cytoplasm of germ cells, Sertoli cells, and in some Leydig cells. Greater Bax protein immunoreactivity was observed in heat-induced apoptotic germ cells. No discernible changes in Bax expression were, however, apparent in Sertoli cells or in susceptible germ cells that do not undergo apoptosis after heat treatment.

## Discussion

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We have previously demonstrated that mild testicular hyperthermia (43° C for 15 minutes) induces stage-specific and germ cell—specific apoptosis resulting in injury to spermatogenesis in both rats and mice (Lue et al, 1999, 2000a, b). In order to test whether heat alone could induce azoospermia or oligozoospermia in nonhuman primates, we applied local testicular heat treatment to monkeys at 43° C for 30 minutes once daily for 6 days. In this pilot study, no sperm was detected in the semen of 2 of 3 monkeys by 6 and 8 weeks after the first heat treatment; the remaining monkey exhibited severe oligozoospermia (8 x $10^6$/ejaculate, 10.89% of pretreatment levels) 6 weeks after the first heat exposure. The sperm count in all 3 monkeys returned to pretreatment levels 12 weeks after heat treatment. Thus, we conclude that mild testicular hyperthermia induces suppression of spermatogenesis leading to oligozoospermia and azoospermia in the non-human primate model. Similar to our earlier studies in rats, the suppression of spermatogenesis induced by heat exposure for 6 consecutive days is completely reversible. Our findings are in contrast to a previous report that showed that daily immersion of the scrota of monkeys (*Macaca radiata*) to 44° ± 1° C for 20 minutes applied 6 days a week for 8 consecutive weeks resulted in atrophy of the germinal epithelium (Venkatachalam and Ramanathan, 1962). The more severe germinal epithelium damage noted in the earlier study could be due to the higher temperature, the longer duration of heat exposure, and the difference in species of monkey used. A description of observations for recovery was not provided in the previous study.

After a transient decrease in serum testosterone levels at 2 weeks, testosterone levels returned to the normal range. Similar transient changes in serum testosterone levels were observed in rats after single heat exposure (Lue et al, 1999). One possible reason for a transient decrease in serum testosterone levels induced by heat treatment may be due to the temporary inhibition of steroidogenic enzymes in Leydig cells (Kuhn-Velten, 1996; Murphy et al, 2001). Serum inhibin B

levels were lower in all 3 monkeys and remained below baseline in 2 of 3 animals after heat treatment, suggesting that heat may temporarily affect Sertoli cell function (Sharp et al, 1999; Ramaswamy and Plant, 2001; Suescun et al, 2001). Our data further support a notion that in general, a good correlation exists between inhibin B levels and the degree of spermatogenic damage, with spermatogenic arrest at the early stages associated with the lowest inhibin B levels (Meachem et al, 2001).

In the present study, using TUNEL assay and electron microscopy coupled with the detection of cleaved PARP as a biological marker of DNA damage (Tramontano et al., 2000), we have demonstrated the involvement of apoptosis after mild testicular hyperthermia. By week 12, spermatogenesis was completely recovered and the incidence of germ cell apoptosis returned to control levels. Our present study demonstrates that apoptosis is the major underlying mechanism of heat-induced (43° C for 30 minutes daily for 6 days) germ cell death in adult monkeys. The precise mechanisms by which germ cells die in response to heat stress are not fully understood (Rockett et al, 2001). We have previously demonstrated that redistribution of proapoptotic Bax protein is the early step in an apoptotic pathway leading to germ cell death induced by mild testicular hyperthermia in rats (Yamamoto et al, 2000). Our present data also suggest that the proapoptotic Bax protein may be one of the proteins responsible for heat-induced germ cell apoptosis in monkeys. We plan to extend this study to include more animals to examine the combination of mild testicular hyperthermia with exogenous testosterone to test our "2-hit" hypothesis that the combination of hormonal treatment such as testosterone implantation (hit 1) and physical agent (heat exposure; hit 2) is more effective in suppressing spermatogenesis than either treatment alone in nonhuman primates. The planned experiments will allow us to determine whether hormonal treatment or physical agents accelerate germ cell death at different stages and by different pathways similar to the rats.

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## Footnotes

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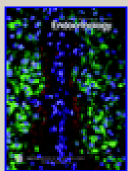

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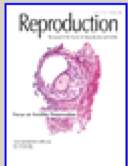

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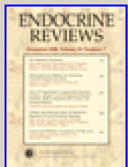

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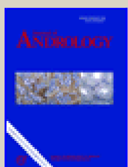

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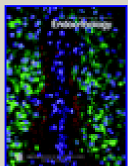

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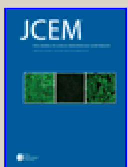

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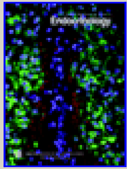

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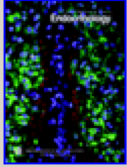

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