

# Mild Hypothermia Induces Apoptosis in Rat Testis at Specific Stages of the Seminiferous Epithelium

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**ABSTRACT:** This study was designed to determine the effects of short episodes of mild hypothermia on cell apoptosis in the testis of the adult rat. Apoptosis was assessed by *in situ* DNA 3'-end labeling in the testes from rats killed 2 hours after cooling (10°C for 30 minutes), quantitated, and compared with spontaneous apoptosis found in control animals. A significantly increased number of dying germ cells appeared after cold treatment at specific stages (stage XIV,  $P < 0.0001$ ; stage XII,  $P < 0.001$ ) of the seminiferous epithelium,

mainly due to dying primary metaphasic spermatocytes ( $P < 0.0001$ ), followed by secondary interphase spermatocytes ( $P < 0.001$ ), and A2 spermatogonia ( $P < 0.05$ ). The highly specific effect of mild hypothermia on germ cell apoptosis suggests that the process is tightly regulated.

**Key words:** Cell death, spermatogenesis, fertility.

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Spontaneous germ cell death in the testis is a matter of interest, but the reasons for death are still unclear. These cell deaths take the form of apoptosis (Blanco-Rodríguez and Martínez-García, 1996), and they were proposed as a way of eliminating cells with chromosomal abnormalities (Clermont, 1962). More recently, the role played by follicle-stimulating hormone (FSH) and testosterone as survival factors controlling germ cell apoptosis has been evidenced (Tapanainen et al, 1993; Billig et al, 1995; Henriksen et al, 1995).

The sensitivity of the seminiferous epithelium to environmental stimuli such as heating, radiation, or cooling may be also a factor promoting the death of germ cells (McDonald and Harrison, 1954; Chowdhury and Steinberger, 1970; Oakberg, 1971), but the effect of low temperatures in germ cell apoptosis has not been previously studied. This study was designed to determine the effects of short episodes of testicular cooling (10°C for 30 minutes) on germ cell apoptosis. For this purpose, apoptosis was assessed by *in situ* DNA 3'-end labeling in the testes from rats killed 2 hours after cooling, quantitated, and compared with spontaneous apoptosis found in control animals. Our findings suggest that the response of germ cells to hypothermia should be tightly regulated, and this may have clinical implications for better understanding etiologies of male infertility.

## Methods

### *Animals and Tissue Preparation*

Ten adult male Wistar rats, weighing 250–300 g, were housed under conventional, controlled standard conditions. The animals were divided into two groups of five. The experimental animals were anesthetized with an intraperitoneal injection of sodium pentobarbital and their scrota exposed to a temperature of 10°C for 30 minutes by immersion in a specially devised water bath. The water bath was equipped with a thermostat regulating the cooling unit and a support allowing the animal body to remain out of the water during cooling of the testes. Normal intact animals were used as control animals.

Two hours after this treatment of mild hypothermia, animals were anesthetized and their testes prepared by making an incision in the tunica albuginea from the proximal to the distal pole. The testes were surgically removed and immersion-fixed in 10% buffered formaldehyde and processed for paraffin embedding.

Animal use and care in this study was approved by the Animal Use and Care Committee of the School of Medicine, Valladolid University.

### *In Situ DNA 3'-End Labeling of Apoptotic Cells*

Labeling of DNA fragmentation in histological sections was done using the Oncor ApopTag nonradioactive detection kit (Oncor, Inc. Gaithersburg, Maryland). Paraffin sections (5 µm thick) were deparaffinized, hydrated, and treated with 20 µg/ml Proteinase K (Boehringer Mannheim, Mannheim, Germany) for 15 minutes at room temperature. DNA 3'-end labeling with non-radioactive digoxigenin-ddUTP (dig-ddUTP) was performed by incubation at 37°C in a humidified chamber for 1 hour. Reaction mixture containing terminal transferase reaction buffer, dig-ddUTP, and terminal deoxynucleotidyl transferase was used following the suppliers' guidelines. DNA strand breaks were revealed after incubation with antidigoxigenin antibody conjugated to peroxidase at room temperature for 30 minutes, as was the

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subsequent detection of enzyme activity using DAB as substrate (as recommended). Finally, slides were counterstained in cresyl violet for 2 minutes at room temperature.

#### Enumeration of Apoptotic Germ Cells and Staging Criteria

Orthogonal sections (5  $\mu\text{m}$  thick) of seminiferous tubules at stages I, II–III, IV, V, VI, VII–VIII, IX, X, XI, XII, XIII, and XIV were examined at 630 $\times$  magnification using a 63 $\times$  (1.4 N.A.) planapochromatic oil immersion objective. Tubule staging was carried out according to the criteria proposed by Russell et al (1990). The labeled cells were identified according to their morphological features and positions within the epithelium and scored, except dying elongated spermatids that, because of their condensed nuclei, were difficult to distinguish with precision. For more accuracy, stages II–III and VII–VIII, as well as all round spermatids, were recorded together. Counts were expressed as a ratio to Sertoli cell nucleoli. For each rat of the control ( $n = 5$ ) and treated ( $n = 5$ ) groups, 10 nonconsecutive sections at every stage were randomly selected. A total of 50 tubules of each stage were studied per group of animals.

#### Data Analysis

Statistical evaluation was performed using the StatWorks software package (1.1 version) on a Macintosh Centris 660AV computer. Comparisons between means were made by a two-tailed Student's *t*-test analysis.  $P < 0.05$  was considered significant.

## Results

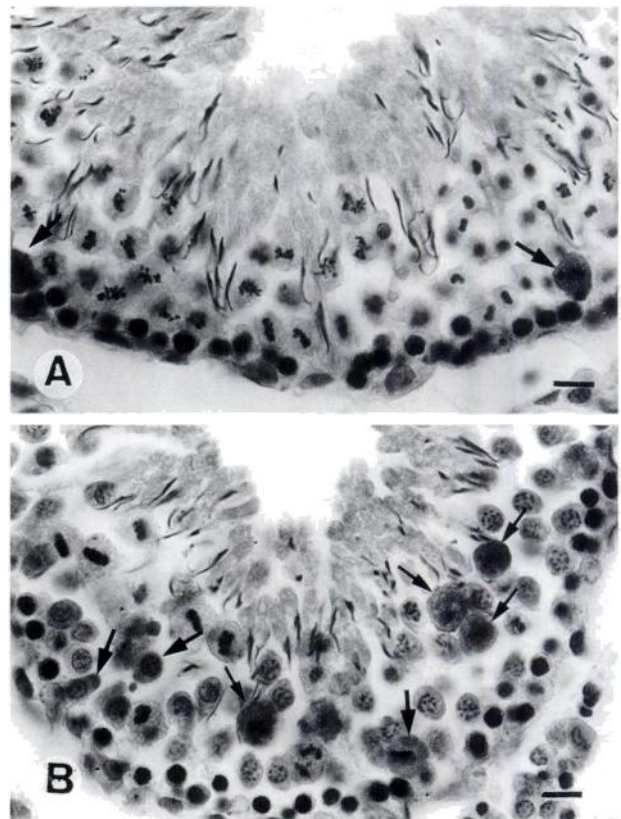
#### In Situ Analysis of DNA Fragmentation

The specific cell types that underwent apoptosis were assessed by direct immunoperoxidase detection of digoxigenin 3'-end-labeled DNA fragments in paraffin-embedded seminiferous tubule (5- $\mu\text{m}$ -thick) sections. Since apoptotic germ cells were scattered among healthy cells and they kept both their main morphological features and right positions, they could be readily identified in both control (Fig. 1A) and experimental rats (Fig. 1B). The number of dying germ cells in all of the cold-treated animals appeared increased with respect to control animals, but immunostained Leydig or Sertoli cells did not appear (Fig. 1).

#### Identification of the Most Vulnerable Stages and Germ Cell Types to Cold Treatment

To identify the specific spermatogenic stages and the germ cell types that were the most vulnerable to cold treatment, we further quantified dying cells at each stage and at each selected maturing step in the testes from control and treated rats.

A significant increased number of dying germ cells appeared after cold treatment at specific stages of the seminiferous epithelium (Fig. 2). The greatest increase was found at stage XIV (more than 10-fold increase,  $P <$

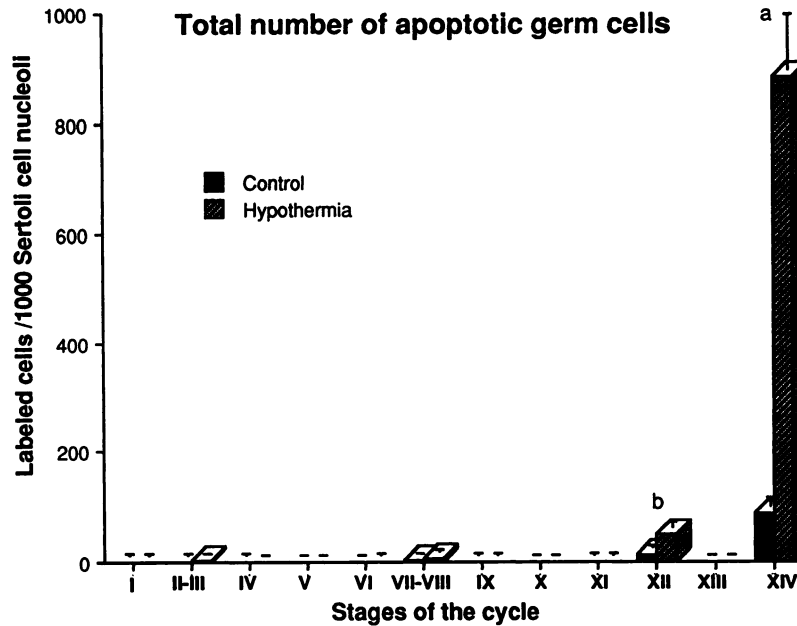


**FIG. 1.** Stage XIV tubules showing immunoreactivity for fragmented DNA in apoptotic primary metaphase spermatocytes (arrows). (A) Control animal. (B) Experimental animal. Magnification 5,200 $\times$ . Scale bar = 10  $\mu\text{m}$ .

0.0001), but a great number of dying cells were also found at stage XII (fivefold increase,  $P < 0.001$ ), where dead cells are also more frequent in control animals (Fig. 2). Increased apoptosis affected nearly all the germ cell types within the epithelium at these stages (A2 spermatogonia and zygotene spermatocytes at stage XII; pachytene, primary metaphase, and secondary interphase spermatocytes at stage XIV). Cell death was the highest at primary metaphase spermatocytes ( $P < 0.0001$ ), followed by secondary interphase spermatocytes ( $P < 0.001$ ). Smaller ( $P < 0.05$ ) increases in dying pachytene spermatocytes at stage XIV and A2 spermatogonia and zygotene spermatocytes at stage XII also appeared (Fig. 3).

## Discussion

Using 3'-end labeling for the detection of apoptotic DNA cleavage and an *in situ* approach, we investigated the effect of mild hypothermia (10 $^{\circ}\text{C}$  for 30 minutes by scrotal immersion in a water bath) on apoptotic DNA fragmentation in the rat testis. The present study demonstrates that this cold treatment induces DNA fragmentation of the



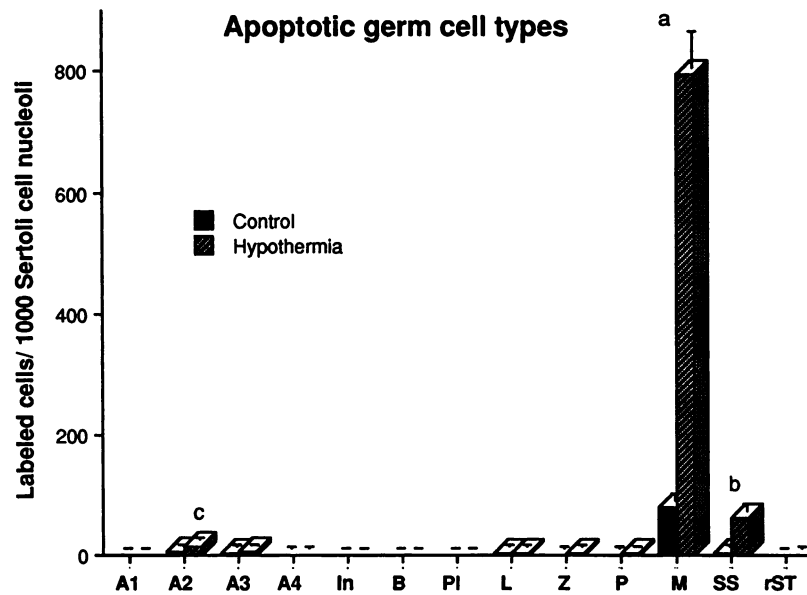
**FIG. 2.** Quantification of stage-specific germ cell apoptosis in control and experimental rats. Dying germ cells were counted at all stages and expressed as a ratio of these cells to Sertoli cell nucleoli. Values are the means  $\pm$  standard error of the mean (SEM) ( $n = 5$  rats). Means with superscripts differ significantly. <sup>a</sup>  $P < 0.0001$ ; <sup>b</sup>  $P < 0.001$ .

germ cells at specific stages of the seminiferous epithelium.

Most mammals have a scrotum, and it is well established that the mild hyperthermia causes disruption of spermatogenesis, leading to infertility (Nelson, 1951; Yavetz et al, 1992). As a consequence, the effects of local heating on the rat testis have been widely studied (Bartlett

and Sharpe, 1987; Chowdhury and Steinberger, 1970). More recently, the triggering of apoptosis has been evidenced as the mechanism of germ cell death occurring in the cryptorchid testes (Shikone et al, 1994).

In contrast, the effects of exposure of testes to local cooling are poorly investigated. Our results demonstrate that mild hypothermia specifically increases apoptosis of



**FIG. 3.** Quantification of degeneration in spermatogonia (A1, A2, A3, A4, In, B), primary preleptotene (PI), leptotene (L), zygotene (Z), pachytene (P), and metaphase (M) spermatocytes, secondary spermatocytes (SS), and round (rST) and elongating (eST) spermatids in seminiferous tubules from control and experimental rats. Values are the means  $\pm$  standard error of the mean (SEM) ( $n = 5$  rats). Means with superscripts differ significantly. <sup>a</sup>  $P < 0.0001$ ; <sup>b</sup>  $P < 0.001$ ; <sup>c</sup>  $P < 0.05$ .

germ cells at stages XII and XIV, whereas there is no increase in apoptotic Leydig, Sertoli, or other germ cell types. This fact indicates that, as with intermediate heat (Allan et al, 1987; Shikone et al, 1994), the testis response to scrotal hypothermia is tightly regulated.

Apoptosis also has been associated with low temperature-mediated disruption of microtubules (Liepins and Youngusband, 1985), and the relationship between cold shock-induced apoptosis and the cell cycle status has been established *in vitro* in several cell types (Soloff et al, 1987). In addition, it is now known that spindle assembly is monitored during the cell cycle checkpoint existing at the metaphase–anaphase border, and when certain errors cannot be corrected, the cell responds with the triggering of apoptosis (Wells, 1996). Therefore, the increase of apoptosis observed after cold treatment in dividing A2 spermatogonia at stage XII and primary metaphase spermatocytes would be easily explained as a direct (or indirect, through the microtubule disruption) action of cold shock on this spindle-assembly checkpoint. In this regard, it is interesting to note that the highest numbers of spontaneously dying germ cells are also found at these steps of germ cell maturation. Nevertheless, this reasoning alone does not seem enough to explain why dying primary pachytene and secondary interphase spermatocytes are also increased at stage XIV. Since it has been evidenced that the sensitivity to cold shock-induced cell death is critically dependent *in vitro* on the serum concentration (Kruman et al, 1992), one explanation could be that this situation might be equivalent *in vivo* with those stages with lower levels of survival factors (Tapanainen et al, 1993) such as stage XIV at which gonadotropins, as well as testosterone, are at low levels (Parvinen, 1982).

As it happens upon heat induction, the synthesis of heat-shock proteins increases drastically after cold shock (Liu et al, 1994), and increased levels of heat-shock proteins have also been detected during apoptotic cell death (Buttayan et al, 1988). Therefore, because heat-shock proteins are expressed in rat testes (Itoh and Tashima, 1991), another interesting possibility is that they might play a role in testis apoptotic cell death. Germ cell apoptosis and male infertility have been evidenced after disruption of the Hsp 70-2 gene (Dix et al, 1996), and immunoneutralization of heat-shock proteins increases the heat-shock induced apoptosis (Riabowol et al, 1987). This suggests that heat-shock proteins may be linked to mechanisms that inhibit apoptosis. Taken together, these data are consistent with the idea that it is very likely that some male infertilities, currently classified as idiopathic, could be caused by disruption of heat-shock proteins with the subsequent increase in germ cell apoptosis leading to oligozoospermia and male infertility.

In conclusion, our results demonstrate the highly spe-

cific response of germ cells to mild hypothermia, indicating that apoptosis induced by low temperature in the testis is tightly regulated. As a consequence, we hypothesize that the disruption of its control might be a main factor causing increased germ cell deaths and oligozoospermia, and this might have clinical implications for understanding male infertility.

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