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Spermatocyte apoptosis, which involves both intrinsic and extrinsic pathways, explains the sterility of *Graomys griseoflavus* × *Graomys centralis* male hybrids

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Abstract. Spermatogenic impairment and the apoptotic pathways involved in establishing sterility of male hybrids obtained from crossing *Graomys griseoflavus* females with *Graomys centralis* males were studied. Testes from *G. centralis*, *G. griseoflavus* and hybrids were compared at different ages. Terminal transferase-mediated dUTP nick-end labelling assay (TUNEL), Fas, Bax and cytochrome c labelling were used for apoptosis evaluation, and calbindin D_{28k} staining as an anti-apoptotic molecule. In 1-month-old animals, spermatocytes were positive for all apoptotic markers, but moderate TUNEL (+) spermatocyte frequency was only found in *G. centralis*. At subsequent ages, the apoptotic markers were downregulated in testes from parental cytotypes, but not in hybrid testes. TUNEL (+) spermatocytes were present at 78% and 44% per tubule cross-section in 2- and 3-month-old hybrid animals, respectively. Pachytene spermatocyte death in adult hybrids occurs via apoptosis, as revealed by high caspase-3 expression. Calbindin was highly expressed in spermatocytes of adult hybrids, in which massive cell death occurs via apoptosis. Calbindin co-localisation with TUNEL or Fas, Bax and cytochrome c was very limited, suggesting an inverse regulation of calbindin and apoptotic markers. Hybrid sterility is due to breakdown of spermatogenesis at the pachytene spermatocyte stage. Both extrinsic and intrinsic pathways are involved in apoptosis of spermatocytes, which are the most sensitive cell type to apoptotic stimuli.

Additional keywords: germ cells, mice, Robertsonian fusions.

Introduction

The genus *Graomys* comprises a South American group of rodents, which are distributed from Paraguay and Bolivia to southern Argentina, and probably southern Brazil (Musser and Carleton 2005; Lanzone *et al*. 2007). These animals exhibit a remarkable Robertsonian (Rb) autosomal polymorphism, which apparently occurs by Rb fusions in a non-random sequence (Zambelli *et al*. 2003). These chromosomal rearrangements resulted in different diploid numbers in individuals that are morphologically indistinguishable. Several species have been described in this genus, but certain confusion about the taxonomic status, phylogenetic relationships and overall distribution was a matter of revision and debate. In Argentina, cytotypes $2n = 34$–38 cohabit in the region known as 'Monte' (central-western area) and individuals $2n = 42$ in the region designated 'Espinal' (central zone), but both distribution areas overlap in borderlands (Theiler and Blanco 1996). Cytogenetic, molecular and reproductive data support the idea that $2n = 42$ is the ancestral cytotype from which the other karyomorphs derived through Rb fusions (Gardner and Patton 1976; Zambelli *et al*. 1994; Theiler *et al*. 1999*a*; Zambelli and Vidal-Rioja 1999). Phylogenetic studies based on cytochrome *b* and D-loop fragments of the mtDNA have shown that individuals with $2n = 41$–42, pertaining to *Graomys centralis* (*G. centralis*), form a separate clade from individuals with $2n = 34$–38, pertaining to *Graomys griseoflavus* (*G. griseoflavus*) (Catanesi *et al*. 2002). Reproductive studies have shown that crosses between $2n = 42$ males and $2n = 36$–38 females produced hybrids, while the reciprocal crosses were unproductive. All hybrid males and 80% of hybrid females were sterile. Histological observations of testes from hybrids heterozygous for two or three Rb fusions indicated that spermatogenesis was blocked at the

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level of primary spermatocytes (Theiler *et al*. 1999*b*). No further description about arrest of spermatogenesis of hybrid males from the genus *Graomys* is present in the literature.

Sterility is commonly observed in inter- or intraspecific hybrids, which may result from chromosomal or genic incompatibilities. However, there is little information available on the mechanisms causing hybrid sterility as well as those involved in determining the apoptotic pathways leading to germ cell death.

In a well known Rb heterozygous mouse model of chromosomal-derived subfertility or sterility (Redi *et al*. 1985; Redi and Capanna 1988; Hauffe and Searle 1998; Castiglia and Capanna 2000; Wallace *et al*. 2002), Merico *et al*. (2003) have found a high percentage of defective seminiferous tubules with massive germ cell death. Using the same Rb mouse model with reduced fertility, we have recently demonstrated an intense apoptosis of germ cells mediated, at least in part, by a mitochondrial apoptotic mechanism. We observed mitochondria relocation close to the paranuclear region of spermatocytes, and Bax and cytochrome c redistribution in metaphase spermatocytes. The redistribution of these apoptotic molecules could be responsible for a cascade of events leading to DNA fragmentation and, consequently, to germ cell death. The high expression of calbindin D_{28k} (CB), a putative anti-apoptotic molecule, in metaphase spermatocytes negative for the apoptotic markers led us to postulate that CB might protect germ cells from apoptotic death (Merico *et al*. 2008). The possibility that the process could be a caspase-dependent or -independent mechanism was not explored, nor were other apoptotic pathways.

The apoptotic mechanisms in germ cells are multifactorial and are not completely elucidated. It is known that apoptosis is a normal process during spermatogenesis, which limits the number of germ cells to ensure that Sertoli cells can provide nutrients for continuous generation of germ cells (Lee *et al*. 2006). The Fas system, involving a set of caspases, has been reported to produce spermatocyte apoptosis in the first round of rat spermatogenesis (Lizama *et al*. 2007). A caspase-independent mechanism triggered by calpain activation is another mechanism proposed to be involved in germ cell apoptosis (Coureuil *et al*. 2006). Members of the p53 family and high $[Ca^{2+}]_i$ seem to be regulators of apoptotic death in male germ cells (Petre-Lazar *et al*. 2007). NF-kappaB signals have shown to be increased in TUNEL (+) germ cells in cryptorchid testis, which suggest that NF-kappaB has some role in germ cell apoptosis (Mizuno *et al*. 2009).

The aim of the present study was to use hybrid males obtained from crossing *Graomys griseoflavus* females with *Graomys centralis* males, which are known to be sterile, to address the following questions: (1) to what extent do the hybrid males exhibit deleterious changes in the seminiferous epithelium, (2) are both intrinsic and extrinsic apoptotic pathways involved in germ cell death and (3) is CB overexpressed in the spermatogenic cells as occurs in the Rb heterozygous mice?

Materials and methods

Animals

Nine male animals from each parental species and their derived hybrids were used to accomplish the study: (1) *Graomys centralis* ($2n = 42$ chromosomes), (2) *Graomys griseoflavus* ($2n = 34$) and (3) hybrids ($2n = 38$) obtained by crossing *Graomys griseoflavus* females with *Graomys centralis* males. The animals (three for each group and each age) were 1, 2 and 3 months old. *Graomys centralis* were captured in the area of Montecristo and Capilla de los Remedios (province of Cordoba, Argentina), *Graomys griseoflavus* were captured in the area of Ñacuñan (province of Mendoza, Argentina) and hybrids were obtained in the laboratory 'Dr Cañas' from the Facultad de Ciencias Medicas, Universidad Nacional de Cordoba, Argentina. The karyotype of each animal was controlled from metaphase preparations obtained from bone marrow (Ford and Hamerton 1956). Body and testis weight were determined in all animals. The size of the testes from hybrids was always very small, thus limiting the amount of biological material for hormonal assays. The weight of each animal increased with age, but there were no weight differences among the three groups of animals at different ages (1-month-old animals, 48 ± 2 g; 2-month-old, 73 ± 2 g; 3-month-old, 82 ± 3 g). Serum testosterone levels were assayed by electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). The animals were maintained according to the Guide for Care and Use of Laboratory Animals. All of them were killed by cervical dislocation; efforts were made to minimise their suffering.

Chemicals

All chemicals were purchased from Sigma Aldrich Co (St Louis, MO, USA) unless otherwise stated.

Histology

The right testis of each animal was fixed in Bouin's fluid before processing for paraffin embedding. Five-micrometre serial transverse cross-sections were made for the subsequent co-localisation analyses. PAS reaction and haematoxylin counterstaining were used to visualise tissue sections and analyse cell morphology.

DNA end labelling of tissue sections (TUNEL)

DNA fragmentation was detected by the terminal transferase-mediated dUTP nick-end labelling assay (TUNEL) employing ApopTag Plus peroxidase *in situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA). The detection of peroxidase activity was performed by using 3,3′-diaminobenzidine (DAB; Zymed Laboratories Inc., Invitrogen, Carlsbad, CA, USA) as a chromogen and the sections were counterstained with 0.5% (w/v) methyl green for 10 min at room temperature. The apoptotic cells were counted at 400× magnification in at least three sections from three animals for each treatment, which was accomplished by two independent researchers in a blinded fashion. Positive and negative controls were also performed. The positive controls were established using the slides contained in the same kit following the manufacturer's instructions. Sections processed without TdT enzyme in the labelling reaction mix were used as negative controls.

Immunohistochemical analysis

Serial sections were processed according to the streptavidin–biotin peroxidase complex method. After deparaffinisation, sections were hydrated and incubated for 10 min in 0.5% (v/v)

H_2O_2 diluted in methanol to reduce endogenous peroxidase activity. The slides were rinsed in PBS and incubated with normal bovine serum at 10% (v/v) in PBS for 10 min to avoid non-specific binding of the primary antibody. Later, the primary antibodies were applied at different dilutions. Bax was localised using a rabbit anti-mouse Bax (P-19 polyclonal antibody, 1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cytochrome c localisation was carried out using a mouse anti-cytochrome c monoclonal antibody (1 : 1000; BD Biosciences PharMingen, San Jose, CA, USA). Fas was detected by using a purified mouse anti-CD95 monoclonal antibody (1 : 500; BD Biosciences PharMingen). CB was localised using a mouse anti-bovine CB monoclonal antibody (clone CB-955, 1 : 1000; Santa Cruz Biotechnology). Bax and Fas antibodies were incubated overnight at 4°C, while cytochrome c and CB antibodies were applied at 37°C for 1 h. Thereafter, the sections were washed in PBS and incubated with appropriate secondary biotinylated antibodies diluted in PBS. Half an hour later the sections were incubated with peroxidase-conjugated streptavidin (Zymed Laboratories Inc.) and developed with DAB. The same procedure but without the primary antibodies was used for the control sections. Bax, cytochrome c and Fas immunoreacted sections were counterstained with haematoxylin. Sections were visualised employing a Leica DM microscope (10×/0.25 and 40×/0.65 N PLAN objectives) and images were obtained with a Leica DC 180 Camera (software Leica IM50 Image Manager; Leica, Cambridge, UK).

Western blot analysis

The expression of Fas, Bax, cytochrome c, CB, procaspase-3 and active caspase-3 proteins was determined by protein extraction followed by western blotting analysis. The left testis of each animal, freed from the albuginea membrane, was homogenised in PBS. The total protein suspension of each testis was centrifuged at 13 000*g* for 10 min at 4°C and the protein concentration of supernatants was determined using the method of Gornall *et al*. (1949). Supernatant suspensions (50 μg protein/sample) were mixed with RIPA lysis buffer (1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate in PBS, containing 1 mM PMSF and 1 mM NaF). The mixture was denatured for 5 min at 95°C and separated in 12% (w/v) SDS-polyacrylamide minigels. Gels containing the separated proteins were immersed in the transfer buffer (25 mM TRIS-HCl, 192 mM glycine, 0.05% w/v SDS and 20% v/v methanol). Nitrocellulose membranes (0.45 μm) were blocked for 1.5 h with 2% (w/v) nonfat dry milk in 0.5 M Tris-buffered saline solution (TBS) and incubated overnight at 4°C with the primary antibodies at 1 : 1000 dilution (Fas, cytochrome c, caspase-3) and at 1 : 500 dilution (CB, Bax). Purified rabbit anti-active caspase-3 monoclonal antibody (BD Biosciences PharMingen) was used to detect not only the active form of caspase-3, but also the pro-enzyme form of caspase-3. After three washings, appropriate biotinylated secondary antibodies were incubated at 37°C for 1 h. Then the blots were washed three times and streptavidin-biotin conjugate (Zymed Laboratories Inc.) was added. Detection was performed using DAB as a chromogen. Monoclonal antibody anti-GAPDH (clone GAPDH-71.1) was used to detect GAPDH as a marker to normalise the relative expression of the other proteins. The band intensities were quantified using KS Lite version 2.0 software (Kontron Elektronik GmbH, Eching, Germany) in order to obtain the relative expression of proteins.

Statistical analysis

Five tissue sections were selected at random from each testis for both the immunohistochemical studies and the TUNEL assay. A cross-section of a tubule was considered apoptotic (TUNEL (+) tubule) when two or more TUNEL (+) cells were found. The correction by Abercrombie (1946) was applied to all cell counts. Two serial sections were treated with two different antibodies (CB and Fas, CB and Bax, CB and cytochrome c) or with CB antibody and TUNEL in order to compare and analyse the co-localisation of the different markers. Two independent operators observed the serial sections and performed the counting under the microscope. Also, the serial sections were captured and properly aligned using Photoshop CS software for colocalisation analysis. Statistical significance among the means of the different groups was assessed by the one-way ANOVA followed by the Bonferroni's *post hoc* test. $P < 0.05$ was considered to be statistically significant.

Results

Hybrids exhibit decreased testis weight and serum testosterone and high apoptosis of germ cells

The three groups of animals (*G. centralis*, *G. griseoflavus* and hybrids) did not show differences in bodyweight, as described under the Materials and methods section. However, the testicular size was much smaller in hybrids as compared with that of *G. centralis* or *G. griseoflavus* at the different ages. Therefore, the testicular weight/bodyweight ratio was lower in hybrids than in the other two groups (Table 1). Serum testosterone levels were also lower in hybrids in comparison with *G. centralis* or *G. griseoflavus*. The hormone values increased from 1 to 2 months of age in *G. centralis* and in *G. griseoflavus*, but remained unaltered in hybrids. These latter animals showed a significant increase in the serum testosterone level one month later; however, the concentrations remained eight times lower than those from animals with parental cytotypes (Table 1). The testicular histology of *G. centralis* and *G. griseoflavus* had a normal appearance while that from 2- and 3-month-old hybrids showed severe germ cell loss (Fig. 1). In 1-month-old *G. centralis*, very few spermatids were visualised in the seminiferous tubule sections, whereas leptotenes were observed in *G. griseoflavus* and early pachytene cells in hybrids (Fig. 1*a*, *e*, *i*). The seminiferous epithelium showed all stages of male germ cell differentiation in 2- and 3-month-old *G. centralis* and *G. griseoflavus* (Fig. 1*c*, *g*). By contrast, spermatogenesis was arrested in 2- and 3-month-old hybrids; almost only meiotic cells with a high degree of degeneration were found (Fig. 1*k*), most of which appeared to be degenerating. To ensure that spermatogenic cell death was indeed apoptoptic, we examined DNA fragmentation by the TUNEL assay, as another apoptotic phenotype. TUNEL staining showed a significant number of apoptotic cells in seminiferous tubules from 1-month-old *G. centralis* (Fig. 1*b*) and from hybrids at 2 and 3 months of age (Fig. 1*l*). Most of the TUNEL (+) cells were pachytene spermatocytes and only

Table 1. Testicular and body weight ratio and serum testosterone from different cytotypes of *Graomys*

Data are expressed as mean ± s.e.m. $^{*}P < 0.05$ *v.* 2- and 3-month-old animals with the same cytotype; $^{**}P < 0.001$ *v.* 1- and 2-month-old hybrids; $^{\dagger}P < 0.001$ *v. G. centralis* at the same age; $^{\ddagger}P < 0.05$ *v. G. griseoflavus* and hybrids at the same age; $^{\S}P < 0.001$ *v. G. centralis* and *G. griseoflavus* at the same age

Cytotype	Age (months)	Testicular weight/ bodyweight 10^{-4}	Serum testosterone (ng mL^{-1})
G. centralis	1	8.70 ± 1.20*	0.75 ± 0.21*‡
G. griseoflavus	1	6.80 ± 1.03*	0.18 ± 0.04*
Hybrids	1	2.70 ± 0.61*†	0.14 ± 0.01
G. centralis	2	24.20 ± 3.72	5.09 ± 0.20
G. griseoflavus	2	28.60 ± 0.63	4.33 ± 0.63
Hybrids	2	11.30 ± 0.79§	0.15 ± 0.05§
G. centralis	3	31.50 ± 1.93	5.02 ± 0.65
G. griseoflavus	3	31.80 ± 2.59	5.07 ± 0.49
Hybrids	3	13.50 ± 1.91§	0.68 ± 0.19**§

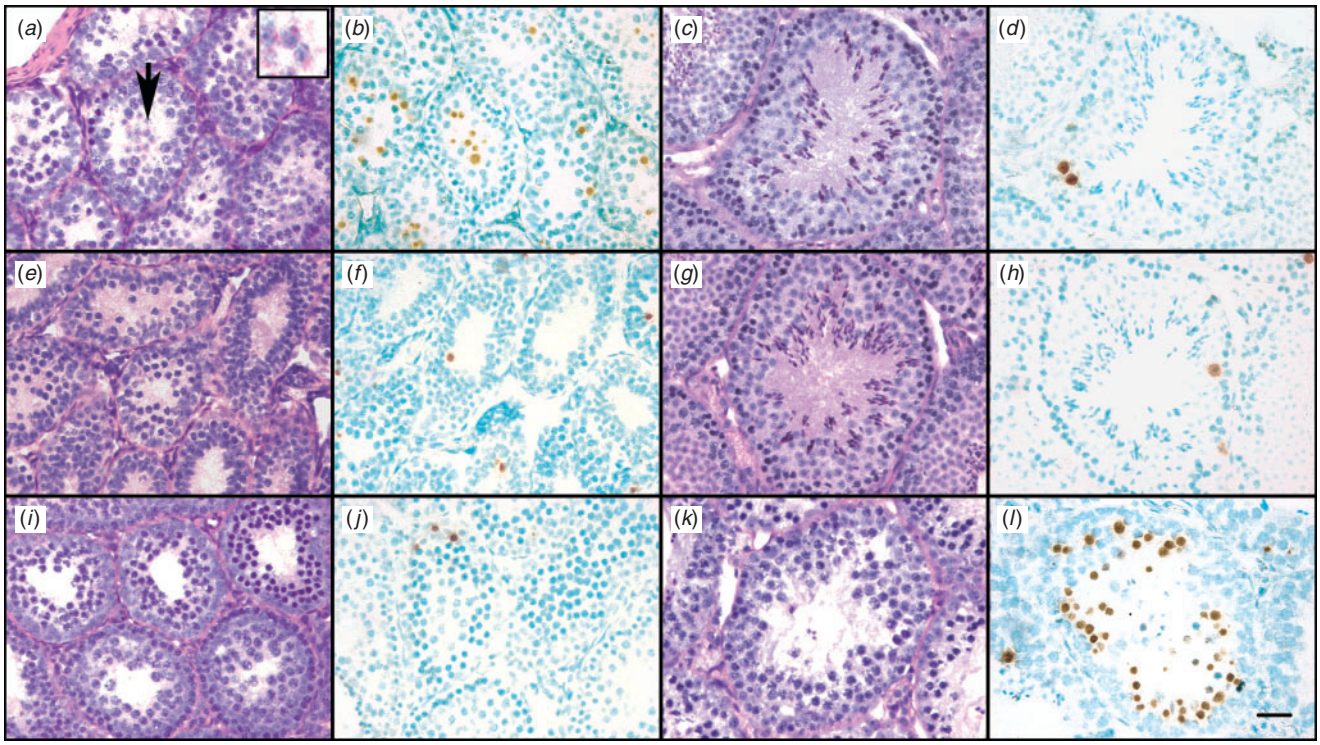

Fig. 1. Seminiferous tubules stained with (*a*, *c*, *e*, *g*, *i*, *k*) PAS-haematoxylin and (*b*, *d*, *f*, *h*, *j*, *l*) TUNEL from (*a–d*) *Graomys centralis*, (*e–h*) *Graomys griseoflavus* and a (*i–l*) *G. griseoflavus* × *G. centralis* hybrid. (*a*, *b*, *e*, *f*, *i*, *j*) 1-month-old animals and (*c*, *d*, *g*, *h*, *k*, *l*) 3-month-old animals. At 1 month of age, a few round spermatids (*a*; arrow and inset) and TUNEL (+) cells (*b*) are present only in the seminiferous tubules from *G. centralis*. At 3 months of age, spermatozoa are present in *G. centralis* (*c*; stage VII of the cycle of the seminiferous epithelium) and *G. griseoflavus* (*g*; stage IV of the cycle of the seminiferous epithelium) but not in hybrid (*k*) seminiferous epithelium, which shows many TUNEL (+) spermatocytes (*l*). Bar = 20 μm.

very few leptotene spermatocytes were also TUNEL (+) in the seminiferous epithelium from 2- and 3-month-old hybrids. No TUNEL (+) cells were observed in the interstitial tissue of testes from any group of animals studied (Fig. 1). The frequency of TUNEL (+) tubules was high in 1-month-old animals of the three cytotypes (Table 2). This frequency became low in *G. centralis* and *G. griseoflavus* at subsequent ages, whilst remained high in hybrids. Taken together, the above data show that the reduced testicular weight in hybrids is paralleled by low serum testosterone levels and loss of spermatogenic cells.

Table 2. Frequency of seminiferous tubule cross-sections positive for the apoptotic markers in different cytotypes of *Graomys*

Data are expressed as mean ± s.e.m. *$P < 0.001$ *v.* 2- and 3-month-old animals with the same cytotype; †$P < 0.001$ *v.* 1- and 3-month-old hybrids; §$P < 0.001$ *v.* other cytotypes at the same age; ‡$P < 0.05$ *v. G. griseoflavus* at the same age; ♦$P < 0.001$ *v.* hybrids at the same age

Cytotype	Age (months)	Percentage of (+) tubule cross-sections per testicular cross-section			
		TUNEL	Fas	Bax	Cytochrome c
G. centralis	1	30.31 ± 2.32*	10.42 ± 0.37*	6.43 ± 0.19*‡	20.34 ± 1.47*♦
G. griseoflavus	1	27.94 ± 2.84*	3.96 ± 3.40	1.54 ± 0.98	11.11 ± 4.02
Hybrids	1	20.89 ± 0.30	2.69 ± 1.63*	2.94 ± 1.17*	6.12 ± 1.42*
G. centralis	2	4.02 ± 1.33	0.00 ± 0.00	1.14 ± 0.71	0.59 ± 0.29
G. griseoflavus	2	11.96 ± 0.61	0.00 ± 0.00	0.83 ± 0.83	3.12 ± 0.44
Hybrids	2	42.62 ± 2.32†§	12.46 ± 1.13§	19.51 ± 1.40§	20.99 ± 0.49§
G. centralis	3	8.62 ± 1.00	0.00 ± 0.00	1.69 ± 0.90	0.69 ± 0.18
G. griseoflavus	3	11.81 ± 2.14	1.61 ± 0.81	0.77 ± 0.55	2.46 ± 0.90
Hybrids	3	26.41 ± 1.81§	11.64 ± 0.40§	17.56 ± 1.55§	19.90 ± 0.79§

Table 3. Frequency of pachytene spermatocytes positive for the apoptotic markers in different cytotypes of *Graomys*

Data are expressed as mean ± s.e.m. *$P < 0.001$ *v.* 2- and 3-month-old animals with the same cytotype; †$P < 0.001$ *v.* 1- and 3-month-old hybrids; §$P < 0.001$ *v.* other cytotypes at the same age

Cytotype	Age (months)	Percentage of (+) pachytene spermatocytes per tubule cross-section			
		TUNEL	Fas	Bax	Cytochrome c
G. centralis	1	36.66 ± 4.34*§	14.75 ± 5.83*	8.61 ± 1.99*	33.93 ± 6.39*
G. griseoflavus	1	0.10 ± 0.10	1.00 ± 0.05	2.12 ± 1.45	11.99 ± 6.15
Hybrids	1	1.40 ± 0.90*	8.40 ± 4.48*	8.09 ± 3.09	37.08 ± 17.87
G. centralis	2	1.81 ± 0.26	0.01 ± 0.00	0.01 ± 0.00	0.25 ± 0.25
G. griseoflavus	2	1.53 ± 0.70	0.01 ± 0.00	0.01 ± 0.00	0.85 ± 0.01
Hybrids	2	78.04 ± 5.80†§	39.27 ± 1.55	39.53 ± 3.27†§	59.87 ± 0.64§
G. centralis	3	1.99 ± 1.44	0.01 ± 0.00	0.01 ± 0.00	0.95 ± 0.52
G. griseoflavus	3	2.07 ± 1.42	0.01 ± 0.00	0.11 ± 0.11	1.13 ± 1.13
Hybrids	3	44.21 ± 2.77§	23.93 ± 6.24	18.82 ± 0.67§	35.97 ± 3.21§

Increased expression of molecules involved in the extrinsic and intrinsic apoptotic pathways in the hybrid testis

Given the evidence that the germ cell death in hybrids occurred via apoptosis, the expression of possible apoptosis-inducing proteins was studied in order to dissect the apoptotic pathway. Fas, a protein involved in the extrinsic apoptotic pathway, and Bax and cytochrome c, molecules involved in the intrinsic apoptotic pathway, were analysed by immunohistochemistry and western blotting in the hybrid testes as compared with those from *G. centralis* and *G. griseoflavus*. Fas (+) tubule cross-sections per testicular cross-section in 1-month-old *G. centralis* were around 10%, a value that decreased markedly with age (Table 2). Fas (+) pachytene spermatocytes were found in the seminiferous epithelium of 1-month-old *G. centralis* cross-sectioned tubules, but Fas expression was not longer detected at subsequent ages (Table 3). A very low frequency of Fas (+) tubule cross-sections was detected in the testes from *G. griseoflavus* at different ages. The percentage of Fas (+) tubule cross-sections in the hybrid testes presented a 4-fold increase at 2 and 3 months of age in relation to the percentage detected in 1-month-old animals (Table 2). Fas was mainly expressed in pachytene spermatocytes. Fas staining was found in ~15% of pachytene spermatocytes per tubule cross-section from *G. centralis*, decreasing to negligible levels at 2 and 3 months. *G. griseoflavus* showed very low Fas expression in pachytene spermatocytes at each age (Table 3). The percentage of Fas (+) pachytene spermatocytes had a 5-fold increase in 2-month-old hybrids in comparison with younger animals, reaching almost 40% of total pachytene spermatocytes per tubule cross-section. The labelling decreased at 3 months, but was still high as compared with 1-month-old animals. A clear age-dependent downregulation of Fas expression of the entire testis from *G. centralis* and *G. griseoflavus* is shown by western blot analysis (Fig. 2). Fas was not downregulated in hybrids, and the protein expression remained high, independent of age. The results indicate that high Fas levels might be associated with germ cell death, mainly with that of pachytene spermatocytes.

The expression pattern of Bax and cytochrome c proteins in testes from the different cytotypes of *Graomys* was quite similar to that of Fas. As shown in Tables 2 and 3 and Fig. 2, Bax and cytochrome c were expressed in seminiferous epithelium from 1-month-old *G. centralis*, decreasing to very low levels at 2 and 3 months. The percentage of Bax (+) tubule cross-sections per testicular cross-section as well as the percentage of Bax (+) pachytene spermatocytes per tubule cross-section

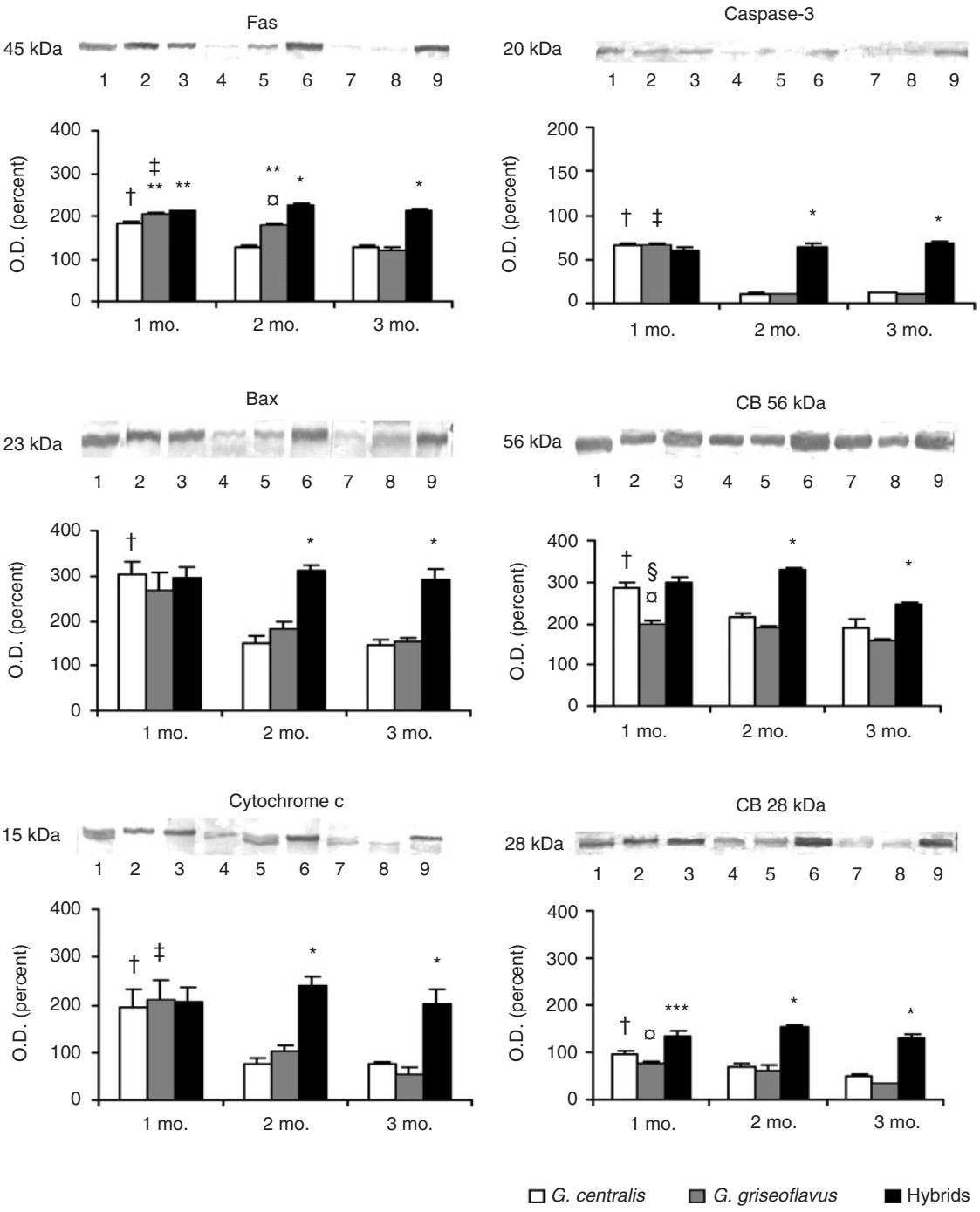

Fig. 2. Representative western blots (upper panels) and quantitative densitometric analysis (lower panels) obtained using anti-Fas, anti-Bax, anti-cytochrome c, anti-caspase-3 and anti-calbindin D_{28k} (CB) antibodies on 50 μg of protein from whole homogenates of testis from *Graomys centralis*, *Graomys griseoflavus* and *G. griseoflavus* × *G. centralis* hybrids at 1, 2 and 3 months of age. Both dimer (56 kDa) and monomer (28 kDa) forms of CB are shown. Each data point represents mean ± s.e.m. ($n = 3$). Data are presented as percentage of GAPDH protein expression (not shown). O.D., optical density. The molecular sizes of the bands are shown on the left of the figure and were determined by weight markers run simultaneously with the protein samples. Lanes: 1, 2, 3, 1-month-old; 4, 5, 6, 2-month-old; 7, 8, 9, 3-month-old animals; 1, 4, 7, *G. centralis*; 2, 5, 8, *G. griseoflavus*; 3, 6, 9, hybrids. $^{\dagger}P < 0.001$ *v*. 2- and 3-month-old *G. centralis*. $^{\ddagger}P < 0.001$ *v*. 2- and 3-month-old *G. griseoflavus*. $^{¤}P < 0.001$ *v*. 3-month-old *G. griseoflavus*. $^{\S}P < 0.05$ *v*. *G. centralis* and hybrid at the same age. $^{*}P < 0.001$ *v*. *G. centralis* and *G. griseoflavus* at the same age. $^{**}P < 0.001$ *v*. *G. centralis* at the same age. $^{***}P < 0.001$ *v*. *G. griseoflavus* at the same age.

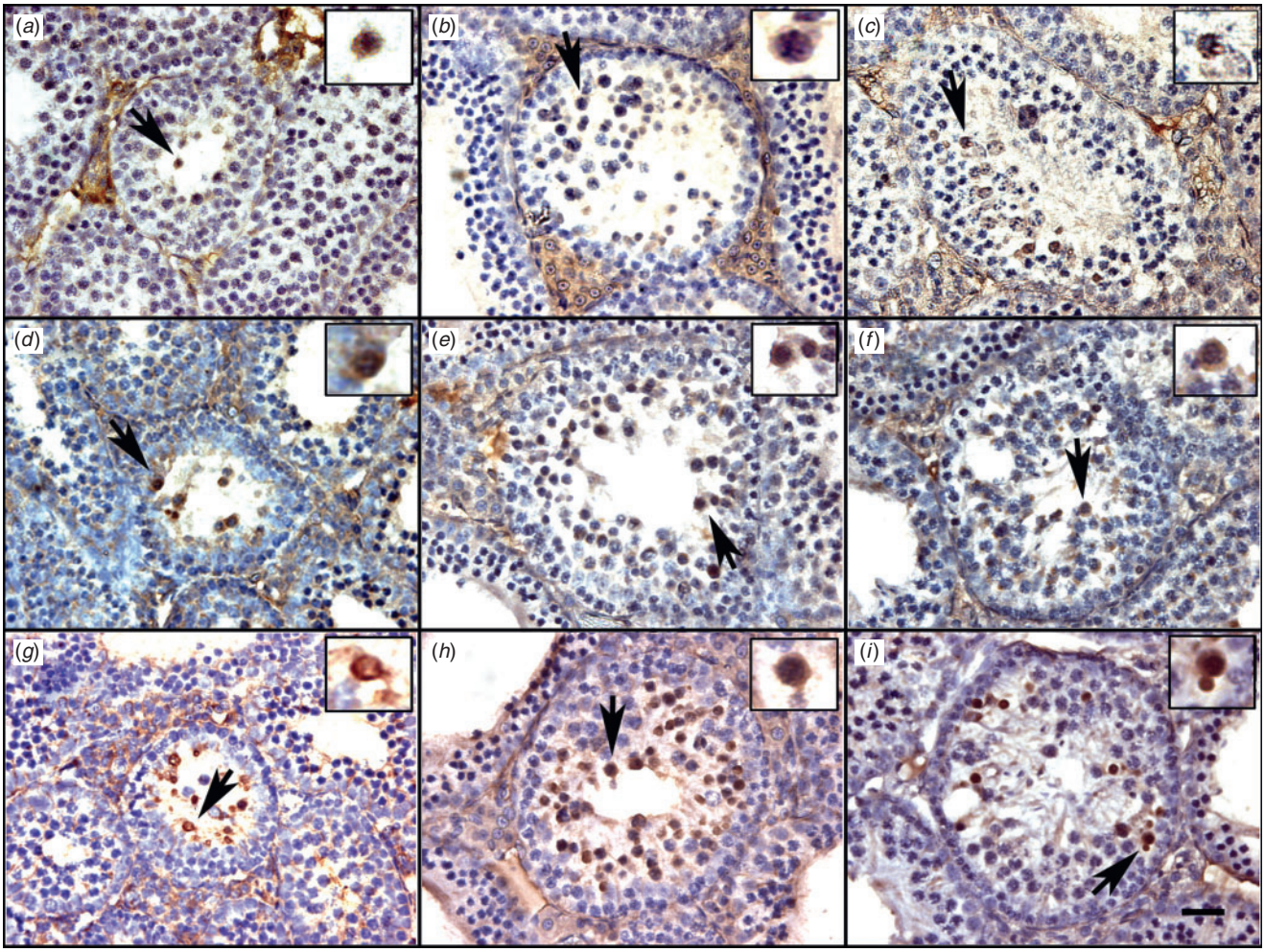

Fig. 3. Seminiferous tubules showing (*a*, *b*, *c*) Fas, (*d*, *e*, *f*) Bax and (*g*, *h*, *i*) cytochrome c labelling from (*a*, *d*, *g*) 1-month-old, (*b*, *e*, *h*) 2-month-old and (*c*, *f*, *i*) 3-month-old hybrids. Cells positive for the apoptotic markers are spermatocytes (arrows) as shown in the insets. Bar = 20 μm.

was low in *G. griseoflavus* at different ages (Tables 2 and 3). Cytochrome c expression was much higher in 1-month-old *G. griseoflavus* than in 2- and 3-month-old animals. A 6-fold increase of Bax expression in tubule cross-sections per testicular cross-section was found in 2-month-old hybrids as compared with younger animals. The magnitude of the enhancement was significant considering that almost 40% of pachytene spermatocytes per tubule cross-section were positive for Bax staining (Table 3). The percentage of cytochrome c (+) seminiferous tubule cross-sections per testicular cross-section was increased 3-fold in 2-month-old hybrids in comparison with that of younger hybrids, reaching almost 60% of pachytene spermatocytes per tubule cross-section. This expression declined moderately one month later. In hybrids, the main expression of these apoptotic molecules occurred in the nucleus and the cytoplasm of pachytene spermatocytes, which means a redistribution of these molecules (Fig. 3). A positive signal for Fas but negative for all the other apoptotic markers analysed was also revealed in Leydig cells from testes of the three cytotypes (data not shown, Fig. 3), indicating absence of cell death.

As occurs with Fas expression, western blot analysis reveals that Bax and cytochrome c expression is downregulated in an age-dependent manner in the entire testis of both *G. centralis* and *G. griseoflavus*. Similarly to Fas expression, hybrids presented lack of this regulation of Bax and cytochrome c expression in the total testis (Fig. 2). High expression of Bax and cytochrome c occurring concomitant with the increased appearance of apoptotic germ cells suggests that the intrinsic pathway would be involved in the mechanism of germ cell death. Given the observation that cytochrome c was redistributed, indicating cytochrome c release from mitochondria, we then examined the expression of the executioner caspase-3. The expression of the pro-enzyme form of caspase-3 was similar in the total testis from all 1-month-old animals, regardless of the *Graomys* cytotype. Pro-caspase expression decreased in 2-month-old *G. centralis* and *G. griseoflavus*, whereas in the hybrids expression remained high and became significantly higher one month later (data not shown). Expression of the active form of caspase-3 decreased markedly with age, both in *G. centralis* and in *G. griseoflavus*. By contrast, expression of the active caspase-3

was high in hybrids, irrespective of age (Fig. 2). Therefore, the results indicate that apoptosis of the pachytene spermatocytes in hybrids involves both the extrinsic and the intrinsic pathways in a caspase-dependent manner.

Most of CB overexpression occurred in pachytene spermatocytes negative for apoptotic markers

CB is a high-affinity calcium-binding protein proposed to be an anti-apoptotic molecule that can prevent or block apoptotic cell death in testis (Merico *et al*. 2008). Its expression was analysed by immunohistochemistry and western blot in testicular tissue from *Graomys* with different cytotypes. As previously found in mice (Merico *et al*. 2008), this protein dimerises in testis showing two bands (28 kDa and 56 kDa). CB expression was detected in pachytene spermatocytes from 1-month-old *G. centralis*, decreasing to negligible levels with age (data not shown). Very low levels of CB protein were observed in pachytene spermatocytes from *G. griseoflavus* at all ages (data not shown). In hybrids, the percentage of CB (+) seminiferous tubules per testicular cross-section had a 4-fold increase in 2- or 3-month-old hybrids as compared with the younger animals, reaching almost 62% of pachytene spermatocytes per tubule cross-section at 2 months (Table 4, Fig. 4*b*). Both the CB monomer and the dimer expression was higher in hybrids as compared with the parental cytotypes (Fig. 2), with its most intense expression concomitant with the highest levels of apoptosis in 2-month-old animals. However, its co-localisation with the apoptotic markers in pachytene spermatocytes was low, indicating that CB localises in different pachytene spermatocytes (Table 4, Fig. 4*a*, *b*).

Discussion

The aim of the present study was to determine the extent of spermatogenic impairment and the cellular and apoptotic mechanisms involved in establishing the sterility of male hybrids ($2n = 38$) obtained from crossing *G. griseoflavus* ($2n = 34$) females with *G. centralis* ($2n = 42$) males. Histological analysis of the seminiferous epithelium of the male hybrids shows that the spermatogenic process is arrested at the meiotic stage, with only rare spermatids present in very few seminiferous tubules. Our findings show that disruption of spermatogenesis at meiosis I, leading to the absence of mature spermatozoa, is the main cause of sterility in hybrids between *G. griseoflavus* × *G. centralis*.

The massive death of pachytene spermatocytes detected in the seminiferous tubules of hybrid males occurs via apoptosis, as revealed by high expression of the executioner caspase-3, which is triggered by both extrinsic and intrinsic pathways. In fact, proteins of both pathways (i.e. Fas, Bax and cytochrome c), as detected by western blot, were maintained highly expressed in the testes of hybrids whereas they were downregulated in parental males of the same age. This lack of downregulation was reflected by a high frequency of Fas, Bax and cytochrome c (+) spermatocytes, as revealed by immunohistochemical analysis, on testicular sections from 2- and 3-month-old hybrids.

Moderate apoptosis of germ cells was detected in spermatocytes from 1-month-old *G. centralis*, but not in *G. griseoflavus* or *G. griseoflavus* × *G. centralis* testes. However, markers of apoptosis were found to be expressed in pachytene spermatocytes in all three cytotypes. Apoptosis of pachytene spermatocytes has been described as a physiological process during the first wave of spermatogenesis (Jahnukainen *et al*. 2004; Lizama *et al*. 2007). The negligible presence of TUNEL (+) spermatocytes in both *G. griseoflavus* and *G. griseoflavus* × *G. centralis* seminiferous tubule cross-sections might be due to a difference in the onset of the first wave of spermatogenesis among cytotypes. In fact, in *G. centralis*, in which post-meiotic cells were already present in the seminiferous epithelium of some tubule cross-sections, a slight, although significant, higher level of testosterone was detected, compared with the other two cytotypes, in which mainly leptotene (in *G. griseoflavus*) and early-pachytene (in hybrids) cells were present in the seminiferous tubules (compare Fig. 1*a* with Fig. 1*e* and *i*). At subsequent ages, the testosterone levels were very similar in both *G. centralis* and *G. griseoflavus*, whose seminiferous epithelia presented all the differentiative steps of the male germ cells, without prominent signs of cell death. In fact, the apoptotic markers were downregulated and the frequencies of germ cells expressing Fas, Bax, cytochrome c or positive for TUNEL staining were extremely low, probably reflecting the physiological apoptosis that occurs in normal testes of adult rodents (Huckins 1978; Allan *et al*. 1992; Kerr 1992; Brinkworth *et al*. 1995; Blanco-Rodríguez and Martinez-Garcia 1996).

On the contrary, apoptosis remained relevant in the seminiferous epithelium of 2- and 3-month-old hybrid males in which 78% and 44% of pachytene spermatocytes were found to be positive for TUNEL staining, respectively. In these animals, spermatogenesis is interrupted at the meiotic pachytene stage, which makes it difficult to determine the stage of the cycle of the seminiferous epithelium at which meiotic cell death occurs. The breakdown of spermatogenesis in *Graomys* hybrids is very severe, with massive germ cell depletion; in fact, only very few spermatids were detected in the seminiferous epithelium. In 2- and 3-month-old hybrids, both the intrinsic and extrinsic markers of apoptosis remained highly expressed, as evidenced by western blots and the frequency of Fas, Bax and cytochrome c (+) spermatocytes. In previous papers, we reported germ cell depletion of both meiotic and postmeiotic stages of differentiation in adult Rb heterozygous mice (Merico *et al*. 2003, 2008). We also showed that the intrinsic apoptotic pathway was involved in germ cell death, although other apoptotic mechanisms were not excluded (Merico *et al*. 2008). Adult hybrids have low levels of testosterone, although we did not find any TUNEL (+) Leydig cells. These low levels of testosterone might contribute to the detrimental effects observed in hybrid spermatogenesis, since testosterone has been shown to be a critical germ cell survival factor (reviewed in Sofikitis *et al*. 2008). The lowering of intratesticular testosterone in a rat animal model resulted in arrest of spermiogenesis and in apoptosis of germ cells (O'Donnell *et al*. 1996; Sofikitis *et al*. 1999; Kim *et al*. 2001). On the contrary, it has been shown that testosterone is able to inhibit *in vitro*-induced apoptosis of human spermatocytes and spermatids (Erkkilä *et al*. 1997). The mechanism by which testosterone withdrawal induces germ cell death has not yet been elucidated. Altered expression of anti-apoptotic Bcl-xl and Bcl-2 molecules was found after intratesticular withdrawal of testosterone in the rat (Show *et al*. 2004). In the present work we did not study the expression of the Bcl-2 anti-apoptotic proteins, but we found high expression of Bax, a Bcl-2 family

Table 4. CB expression and co-localisation with the apoptotic markers studied in hybrid males between *Graomys griseoflavus* × *Graomys centralis*

Data are expressed as mean ± s.e.m. **P* < 0.001 *v.* 2- and 3-month-old; ***P* < 0.05 *v.* 1- and 3-month-old

Age (months)	Percentage of CB (+) seminiferous tubules per testicular cross-section	Percentage of CB (+) pachytene spermatocytes per tubule cross-section
1	5.26 ± 1.66*	23.47 ± 11.56
2	23.40 ± 0.54	61.93 ± 5.26**
3	23.15 ± 1.70	33.59 ± 1.39

Age (months)	Percentage of CB (+) pachytene spermatocytes per tubule cross-section showing co-localisation			
	CB/TUNEL	CB/Fas	CB/Bax	CB/cytochrome c
1	0.00 ± 0.00	1.66 ± 1.66	1.94 ± 1.05	10.86 ± 5.45
2	14.28 ± 2.30**	4.71 ± 1.28	9.44 ± 1.25**	15.20 ± 5.46
3	6.44 ± 0.05	0.18 ± 0.18	2.59 ± 0.68	4.20 ± 0.45

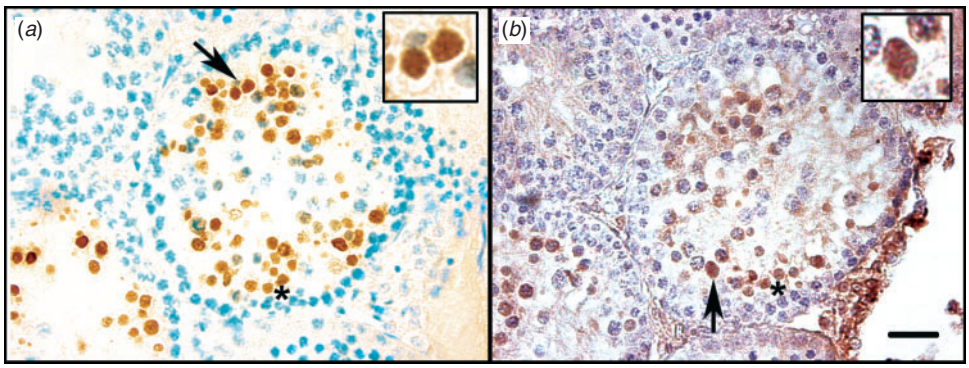

Fig. 4. Seminiferous tubules with (*a*) TUNEL and (*b*) calbindin D_{28k} labelling from 2-month-old *G. griseoflavus* × *G. centralis* hybrid. Most cells positive for TUNEL and calbindin D_{28k} (CB) are spermatocytes (arrows) as shown in the insets. The asterisk marks co-localisation of CB and TUNEL labelling of a spermatocyte. Most TUNEL (+) cells are CB (−). Bar = 20 μm.

member known to block the ability of Bcl-2 to inhibit apoptosis (Oltval *et al*. 1993; Chittenden *et al*. 1995), in hybrids of all ages. Conversely, in adult parental cytotypes Bax expression is downregulated.

Fas was highly expressed in adult hybrids. When it is activated, it promotes processing and stabilisation of caspase-8 (Scaffidi *et al*. 1998; Sánchez-Gómez *et al*. 2003; Henkler *et al*. 2005). Active caspase-8 can proteolitically activate caspase-3, -6 or -7 leading to cell death due to the degradation of many cellular proteins (Riedl and Shi 2004). In mouse, rat and human, the Fas system has been implicated as a possible key regulator of germ cell apoptosis in physiological and injured testes (Lee *et al*. 1997; Pentikäinen *et al*. 1999; Koji *et al*. 2001; Celik-Ozenci *et al*. 2006; Lizama *et al*. 2007). In particular, Fas has been proposed to play a central role in determining pachytene spermatocyte apoptosis during the first wave of spermatogenesis in the rat (Lizama *et al*. 2007). We also found Fas expression in *G. centralis* and to a lesser extent in hybrid spermatocytes of one month of age, confirming the extrinsic pathway to be involved in apoptosis at the onset of the process.

Both intrinsic and extrinsic pathways converge on caspase-3, which was found to be highly expressed in testes from 1-month-old males from any cytotype and in adult hybrids, in which a high frequency of TUNEL (+) spermatocytes was present. We describe for the first time the involvement of both the intrinsic and extrinsic pathways in determining apoptosis in the testes of adult hybrid animals derived from the mating of two different cytotypes of the genus *Graomys* and not induced by testis injury, either chemical or physical.

To maintain testicular homeostasis, pro-survival and pro-apoptotic molecules work together, regulating the extent of apoptosis to produce gametes of high quality (Mishra *et al*. 2006). We have studied the expression of CB, an anti-apoptotic molecule, which has been suggested to protect different cell types against apoptotic cell death induced by both calcium-independent (by the inhibition of caspase activity) and calcium-dependent pathways (Christakos and Liu 2004; Choi *et al*. 2008). Here we show that CB is overexpressed in pachytene spermatocytes from hybrid males, confirming that CB is present in the same cell type that undergoes DNA fragmentation. However, not all pachytene spermatocytes overexpress CB; it appears that a subgroup of these cells are CB (+). Similar results were reported in Rb heterozygous mice, in which CB was found to be expressed in metaphase spermatocytes, the cell type in which apoptosis takes

place (Merico *et al*. 2008). Thus, CB overexpression seems not to be dependent on the cell type. However, in both *Graomys* hybrids and Rb heterozygous mice, the frequency of cells that show simultaneous apoptosis and CB expression is very low. In *Graomys* hybrids, 62% or 34% of cells were CB positive, whereas only 14% and 6% showed co-localisation with TUNEL in 2- and 3-month-old hybrids, respectively. Thus, the expression of CB and that of the apoptotic markers seems to be inversely regulated. The same pattern of expression has been found by Lema Tomé *et al*. (2006) in a model of induced neuronal apoptosis in rats. These authors described immunoreactivity for activated caspase-3 in layers IV/V, between areas of high CB or calretinin expression. Also, in the caudate putamen, activated caspase-3 did not invade zones of intense CB immunoreativity. However, it remains to be elucidated if the expression of CB represents a response to a surveillance mechanism for controlling cell death before the onset of or during the apoptotic process.

This is the first study that describes (1) the timing of the onset of spermatogenesis in *G. centralis* and *G. griseoflavus*, useful knowledge in conservation biology studies and (2) the cellular and apoptotic mechanisms that are involved in determining the spermatogenetic breakdown in hybrids between the ancestral $2n = 42$ cytotype and $2n = 34$ cytotype derived through Rb fusions. We found that pachytene spermatocytes are the most sensitive cell type to apoptotic stimuli driven by both the extrinsic and intrinsic pathways. Here we show that in *G. griseoflavus* × *G. centralis* hybrids a breakdown of spermatogenesis occurs at the meiotic stage of germ cell development, thus accounting for their sterility.

The present study contributes to the knowledge of the cellular basis of spermatogenesis breakdown and the molecular mechanisms involved in germ cell apoptosis, which are important for understanding some aspects of male infertility.

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