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# AGEING INDUCES APOPTOSIS AND INCREASES HSP70 STRESS PROTEIN IN THE EPIDIDYMIS OF *Octodon degus*

## EL ENVEJECIMIENTO INDUCE APOPTOSIS Y AUMENTO DE LA PROTEÍNA HSP70 EN EL EPIDÍDIMO DE *Octodon degus*

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**SUMMARY**: Apoptosis has been largely analyzed in the testis. Nevertheless, the epididymis has been scarcely studied. We analyzed the number of apoptotic cells in the different regions (caput, corpus and cauda) of the epididymis of the South American rodent *Octodon degus* both young and senile. Apoptosis was identified using the TUNEL method which detects *in situ* DNA fragmentation. Apoptosis was detected in the principal cells of the epididymal epithelium. The caput epididymis was the region more affected. The caput of young animals showed that 0.32/1000 cells were apoptotic in contrast to 5.1/1000 of senile animals. Also in the cauda epididymis apoptosis is increased with age, appearing 0.14/1000 and 3.9/1000 in young and senile animals, respectively. On the other hand, we used a immunocytochemical method to localize the stress protein HSP70. HSP70 appeared notably increased in the principal cells of the cauda epididymis of senile animals. Changes in the epididymal epithelium are probably due to the low androgen levels existing in senile animals and are a region dependent phenomenon.

KEY WORDS: 1. Androgens; 2. Apoptosis; 3. Epididymis; 4. Ageing; 5. Heat Shock Proteins.

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## INTRODUCTION

The effect of ageing on the mammalian male reproductive organs has been principally analyzed in the testes. Spermatogenesis and steroidogenesis decrease with old age (Levy *et al.*, 1999; Zirkin & Chen, 2000), and it has been shown that apoptosis increases with age producing an accelerated germ cell loss (Wang *et al.*,1999; Kimura *et al.*, 2003). This changes were related to the fall in androgen levels (Steiner *et al.*, 1984; Gooren, 1998) and/or to the increase in oxidative stress in the tissue (Samanta *et al.*, 1999). In the epididymal epithelium, some segment-specific changes occur at the histological and biochemical levels. These changes include some features which are characteristic of ageing, such as accumulation of lipofuscin granules, a notorious increase in the thickness of the basement membrane, changes in the number of halo cells (Serre & Robaire, 1998), and also modifications in the junctional complexes between epithelial cells (Levy & Robaire, 1999). In addition, changes in the expression of genes related to

oxidative stress in the epididymis due to age have also been described (Viger & Robaire, 1995
and Jervis & Robaire, 2002; ). Male accessory sex glands also experience changes due to aeging: the secretory activity of the ventral prostate decreases and in the prostatic cells, supra and paranuclear pleiomorphic lysosomes can be observed (Zirkin & Strandberg, 1984).

Apoptosis is a well known process that has been associated with ageing (Warner, 1999; Higami & Shimokawa, 2000). It is defined by a set of morphological and biochemical changes at different cellular levels (Wyllie *et al*., 1980) and the result is a physiological elimination of unwanted cells, leaving the surrounding tissue untouched. Apoptosis is triggered by a variety of stimuli and it is widely accepted that it plays a physiological role in the life cycle of many organs, including the gonads (Hsueh *et al*., 1996). In relation to ageing, apoptosis has a primary negative effect by destroying essential and often irreplaceable cells, but it also acts to eliminate dysfunctional cells and protect the organs against cancer or hypertrophia (Warner *et al*., 1997).

On the other hand, heat shock (or stress) proteins (HSPs) have been amply analyzed in the last years (Georgopoulos & Welch, 1993). HSPs and particularly HSP70 functions as molecular chaperones, assist in the folding and transport of proteins and their assembly into complexes, and they protect cells of stress or nocive conditions (Georgopoulos & Welch, 1993). HSP70 has been analyzed in different testicular cells, nevertheless analysis on their presence in epididymal cells has ben scarcely done (Eddy, 1999; Guo *et al*., 2001 ). Furthermore, HSPs are recognized regulators of apoptosis (Beere & Green, 2001).

Analyses on apoptotic phenomenon in the epididymis have shown that orchidectomy induces apoptosis in the epididymis (Fan & Robaire, 1998). Also a notorious increase of apoptosis has been described when the temperature of the organ is artificially increased in 5-7C using a surgical procedure (Jara *et al*., 2002). In this case apoptosis was region specific and affects only the principal cells in the proximal region of the cauda. Moreover, in the mouse, it has been shown that ageing induced an increase of apoptosis in both the epididymis and in ventral prostate epithelium (Jara *et al*., 2003). The changes observed were related to a fall in testosterone levels and a discrete decrease in androgen receptors (Jara *et al*, 2003). In the present report we show the changes induced by ageing in the number of apoptotic cells and in the presence of the heat shock protein HSP70 in the epididymis of the South American rodent *Octodon degus.*

## MATERIAL AND METHOD

Male *Octodon degus* were obtained from the colony of the University of Chile. Animals were housed at constant temperature and in a 10L:14D light cycle. This light period was the stimulatory reproductive cycle for *Octodon degu*. Animals were fed with commercial pellets and water *ad libitum*. We employed two groups of animals. Group 1 was formed by 5 young (6 months-old) animals and Group 2, by 6 old (4.5-5.0 years old) males. The animal protocol used was covered by the law 223/88 on Animal Protection of Spain, and the European Union Agreement about Vertebrate Animal Protection (3/18/1986) and has been approved by the CSIC ethical committee.

Animals were anesthetized using ether. After the animals were bled to death, the epididymis was isolated and dissected free of fat. For fixation, pieces of the three different regions of the epididymis, caput, corpus and cauda, were immersed in 2% paraformaldehyde in phosphate-buffered saline (PBS), and kept for 1-2h or overnight at 4C. Samples were washed in PBS and used for cryosections. Thin sections (5-10 µm) were obtained using a cryostat and were mounted on polylysine-coated slides. For detection of DNA fragmentation *in situ* we employed the TUNEL (Terminal transferase dUTP nick-end labeling) procedure. The detection kit used was supplied by Roche (Basel, Switzerland), and used following the manufacturer's instructions. Slides were covered using a solution of PBS containing 0.1% of Vectashield (Vector Labs. Burlinganme, Illinois) to prevent fluorescence loss and 10 mM of Hoechst 33342 (Sigma, St. Louis, MO) for DNA stain.

HSP70 was analyzed using an immunocytochemical procedure. Samples of the tissues were sectioned using the cryostat. The sections (5-10 µm) were fixed for 10 min in an ice-cold mixture of acetone and methanol (1:1) and then air dried and stored frozen until use. The non-specific binding sites were blocked by incubation for 1h (RT) in 3% BSA in PBS. The primary antibody, an anti-HSP70 was purchased from Amersham (Amersham Biosc. Ltd. Buckinghamshire, England) and used overnight at 4C at a concentration of 2 µg/ml. After several washings an anti-mouse IgG conjugated to FITC diluted 1:200 in PBS was used as the secondary antibody. After washings, slides were covered with the Vectashield/Hoechst 33342 solution.

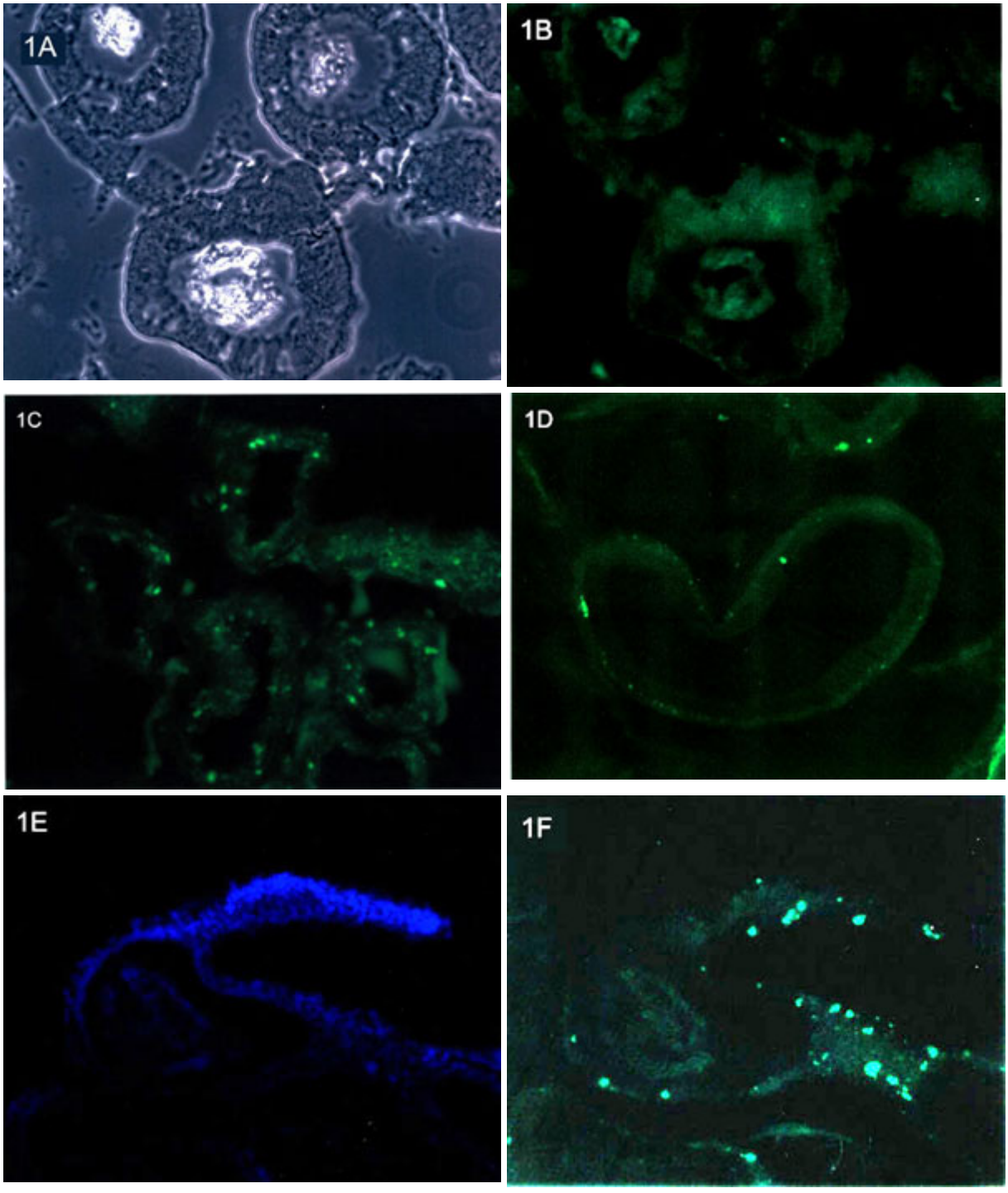
The positive reaction of the TUNEL and immunocytochemistry was visualized under fluorescence using the appropiate filters. Images were captured using a microscope equipped with a CCD system (200A, Polytronics, Emsworth, United Kingdom). A Bio-Rad MRC-600 laser confocal microscope was also employed.

In all cases controls were employed and they were tissues which were treated with buffer , or in the case of immunocytochemistry, omitting the primary antibody.

The percentages of apoptotic cells were determined by counting randomly selected areas with at least 1,000 cells per animal. A minimun of 4,000 cells per group was scored.

**RESULTS**

Apoptotic cells were observed in the different regions of the epididymides of young and old animals. The use of the TUNEL technique allowed us to assess that cells undergoing apoptosis were epithelial cells, and they probably represented principal cells (Figs. 1 C, D, F ). The apoptotic cells appeared ramdomly distributed through the epithelium (Figs 1 C, D, F). Nuclei staining permits to know the cell number and to compare with the positive TUNEL cells which showed a green fluorescence (Figs. 1 E,F). In young animals apoptotic numbers were very low in comparison to the numbers scored in samples from old animals. In the caput epididymis of young animals the 0.32 /1000 of cells were positive to the TUNEL (Figs 1 A, B), nevertheless in the caput of old animals there were 5.1/1000 (Fig 1 C). In the cauda epididymis the increase of apoptosis was also notorious: in young animals only the 0.14/1000 was positive (Fig 1 D), but in old animals was the 3.9/1000 (Figs 1 E,F). The corpus epididymis showed very low numbers (about 0.12/1000-0.22/1000) in both young and old males.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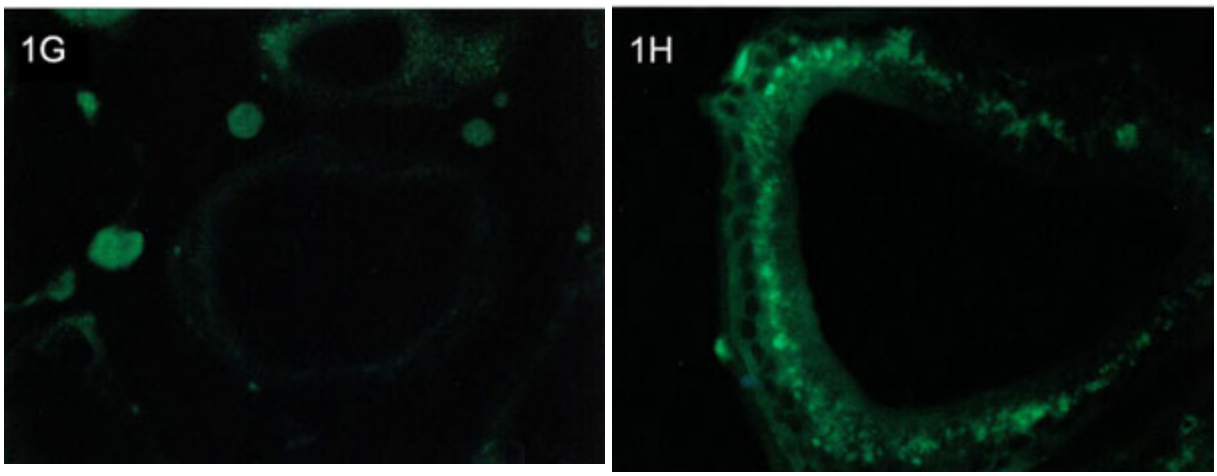


Fig. 1. A and B) Show the phase contrast (A) and fluorescence (B) images of the caput epididymis of a young male. No apoptotioc signs are observed in B. C) Caput epididymis of an old male observed after TUNEL technique. Numerous epithelial apoptotic nuclei can be seen. D) Cauda epididymis of a young male after TUNEL technique. Only two fluorescent nuclei can be observed. E) and F) Section of the cauda epididymis of an old male observed after Hoescht staining for observation of the nuclei (E) and the same showing the TUNEL fluorescence (F). A notorious increase of fluorescent nuclei can be seen if compared to Fig. 1 D. G) and H) HSP70 location in caput (G) and in the cauda (H) of an old male. In the cauda a striking fluorescence is observed in the mid region of the cytoplasm of principal epididymal cells. (Figs A, B, C, D, E, F : 240 X; 1G : 400 X; 1H : 340 X).

Immunochemistry for detection of HSP70 showed that a scarce fluorescence appeared in the epithelium of the epididymis of young animals. Fluorescence appeared as some scattered granules located in the cytoplasm of the principal cells of the cauda epididymis (Fig.1 G). Nevertheless the number of fluorescent granules representing HSP70 was notably increased in old animals (Fig. 1 H). Fluorescence was located in the mid region of the cytoplasm (Fig. 1 H). Control preparations for both TUNEL and HSP70 immunocytochemistry were negative.

## DISCUSSION

Ageing seriously affects reproduction in the male and brings involutive changes in the testes. Apoptosis has been shown to play a role in these changes (Wang *et al.* and Kimura *et al.*). The present results showed that not only the gonads but also the epididymis undergo an increase in apoptotic rates induced by age. The present results using TUNEL compared to the negative results of the controls indicate that positive cells are apoptotic. The changes in the epididymis are not surprising, since the epididymis depends on androgens and testicular factors, and it may well be reflecting the changes in the testes. A regional response was also evident in the epididymis of castrated rats (Adams, 1984). This was probably produced as a reflection of the differences in androgen concentration in the luminal fluid along the epididymis (Fan & Robaire, 1998). The same situation may occur in other species as the rat and mouse, since the old testes in the rat secrete much less testosterone (Turner *et al.*, 1984) and RIA analyses undertaken in the mice showed a considerable decrease in androgen content (Jara *et al.*, 2003). Moreover, there was also a regional response. The most affected region of the epididymis is the caput, and apoptotic indices were higher in the caput and cauda epididymis than in the corpus. The present results are similar to those observed in the epididymis of the aged mouse in which apoptotic indices were also higher in the caput than in the other regions (Jara *et al*, 2003). This characteristic clearly

indicates that the phenomenon is region dependent. Regional diferences in the epididymis has been observed in various cases in which a physiological response of the different segments have been analyzed (Abe *et al.*, 1983 and Serre & Robaire). For example it is well known that the caput is the region most sensible to the action of androgens (Turner *et al.*). This regional differences are probably due to the different androgen levels received from the testis, to the different physiology of principal cells and to the molecules secreted and reabsorbed by this epididymal region (Cooper, 1986). In the case of *Octodon degus* previous analyses showed that testosterone decreases a 91.2% in the plasma and 37.3% in the testis in the senile *Octodon degus* (Bustos Obregón & Ramírez, 1997). That the androgen withdrawal was ultimately responsible for the induction of apoptosis in the epididymis was supported by the fact that androgen supplementation abolished the age-induced apoptosis in all epididymal segments (Jara *et al.*, 2003).

On the other hand it has been pointed out that oxidative stress could be the link to apoptosis because it can induce apoptosis in a variety of cell types (Brunk & Terman, 2002; Curtin *et al.*, 2002), so it is possible that the fall in testosterone can induce a joint increase in oxidative stress and apoptosis. In fact that deserves some comment, is that testosterone supplementation was able to suppress the deleterious effects of old age in the male tract, at least regarding apoptosis in the epididymis (Jara *et al.*, 2003).

The relation of HSPs with the apoptotic phenomenon has been described (Beere & Green, 2001). HSPs appear as elements which increase resistance to apoptosis in different cells and tissues (Samali & Cotter, 1996). In the case of the mouse epididymis subjected to abdominal temperature in which apoptotic indices appeared notably increased (Jara *et al.*, 2002), no changes were detected in the level of HSP70. In our case, HSP70 appeared clearly increased as noted in the immunocytochemical preparations. This increase was principally observed in the cauda epididymis. This fact, as for the case of apoptosis, revealed that this is a region-specific phenomenon. It seems to be obvious that the increase of this protein in the principal cells of the epididymis represent a reaction to the ageing changes, and would increase the resistance to apoptosis as mentioned in other cell systems (Samali & Cotter).

An interesting fact is that principal cells of the epididymis are the principal responsable of the epididymal function related to spermatozoon maturation (Bedford, 1975; Cooper). It is well known that the secretion of epithelial cells, and particularly of those from the caput segment, becomes attached to the spermatozoon surface, and probably control the cell to cell attachment occurring between gametes during fertilization (Cooper). Then, the presence of apoptosis in the epididymal epithelium could be a probable cause of disfunction of this organ. This fact is obviously related to the loss of fertility occurring in old males (Bustos Obregón & Ramírez).

In summary, old age induces apoptosis in the epididymis of the rodent *Octodon degus*, which seems to be caused by the fall of testosterone levels and is probably related to an increase in the oxidative stress in the tissues. Furtehermore, HSP70 is also induced by ageing in the epididymal epithelium. More detailed analyses are needed to elucidate the precise molecular mechanisms involved in there age-induced changes.

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La apoptosis ha sido muy estudiada en el testículo, no así en el epidídimo. Se analiza el número de células apoptóticas en las diferentes regiones epididimarias (cabeza, cuerpo, cola) del roedor sudamericano *Octodon degus* joven y senil. La apoptosis se identificó con la técnica de TUNEL que detecta fragmentación del ADN y se la observó en las células epiteliales principales. La cabeza fue el segmento más afectado, con 0.32/1000 células apoptóticas en los animales jóvenes vs 5.1/1000 en seniles. También en la cola la apoptosis aumenta con la edad, con 0.14/1000 y 3.9/1000 en jóvenes y seniles, respectivamente.

Por otra parte, la detección inmunohistoquímica de la proteína de estrés termico HSP-70 mostró que ésta aumenta notoriamente en las células principales de la cola epididimaria en animales seniles. Los cambios en el epitelio epididimario se deben probablemente a los bajos niveles de andrógenos del animal senil y son dependientes de la región epididimaria analizada.

PALABRAS CLAVE: 1. Andrógenos; 2. Apoptosis; 3. Epidídimo; 4. Envejecimiento; 5. Proteínas de choque térmico.

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