Testicular histomorphometry and the proliferative and apoptotic activities of the seminiferous epithelium in Syrian hamster during spontaneous recrudescence after exposure to short photoperiod

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19 **Contents**

20 Syrian hamsters are photoperiodic rodents in which reproduction, including testicular
21 function, is stimulated by long photoperiod exposure and curtailed by exposure to a
22 short photoperiod. The objectives of this study were to characterize the testis
23 histomorphometrically and to determine the role of the proliferation and apoptosis
24 phenomena in the recovery of the seminiferous epithelium during spontaneous
25 recrudescence after exposure to short photoperiod. The study was performed using
26 conventional light microscopy, proliferating cell nuclear antigen and terminal
27 deoxynucleotidyl transferase (TdT)-mediated dUTP *in situ* nick end labelling staining,
28 image analysis software, and transmission electron microscopy in three recrudescence
29 groups: initial recrudescence (IR), advanced recrudescence (AR), and total
30 recrudescence (TR). Morphometrically, the results pointed to the gradual recovery of
31 the testicular and tubular volumes, as well as of the seminiferous epithelium. Among the
32 IR and AR groups, the increase in testicular and tubular volumes was accompanied by
33 an increase in tubular diameter and length, with an increase in interstitial volume. From
34 AR to TR, there was an increase in the tubular and total volumes, but, in this case, with
35 a gradual increase in tubular diameter. Recovery of the seminiferous epithelium was
36 accompanied by changes in apoptosis and proliferation activities. The first decreased
37 half way through the process and the second remained higher than the control levels
38 throughout the recrudescence stage. Ultrastructurally, alterations in the spermatozoa
39 were observed, which indicated that spermiogenesis was not yet completely normal. In
40 conclusion, spontaneous testicular recrudescence in Syrian hamster comprises two
41 histomorphometrical phases, the first related to an increase in tubular length and
42 diameter and interstitial volume, and the second depending principally on the gradual
43 increase in tubular diameter. The restoration of the seminiferous epithelium is due to

apoptosis reaching normal values in the AR group accompanied by higher proliferative activity than that observed in the Control group.

47 **1. INTRODUCTION**

48 The hamster, a rodent commonly used in the laboratory as a model of seasonal
49 reproduction, shows changes in reproductive function mainly associated with changes in
50 the amplitude of hours of light, or photoperiod (Bronson et al., 1999). The duration of
51 melatonin secretion each day is directly proportional to the length of the night, while
52 and long-term melatonin release induces gonadal regression**.** At the same time, this
53 increase in melatonin inhibits the pituitary hormones, FSH, LH and prolactin, followed
54 by a decrease in testicular testosterone synthesis (Bronson et al., 1999; Berndtson &
55 Desjardins, 1974; Kelly et al., 1994). As a consequence of this reduction in
56 gonadotropins, the testis atrophies or undergoes regression. In the case of the Syrian
57 hamster (*Mesocricetus auratus*), testicular regression involves decreased testicular
58 weight and volume, and lower rates of androgen synthesis and spermatogenesis
59 (Berndtson & Desjardins, 1974; Seco-Rovira et al., 2015). This loss in spermatogenesis
60 involves a depletion of the seminiferous epithelium with the arrest of spermatogenesis
61 in primary spermatocytes (Hikim et al., 1988). Once the maximum degree of regression
62 is reached, and without any change in the photoperiod, the hypothalamic-pituitary-
63 gonadal axis stops responding, becoming insensitive to melatonin and, as a
64 consequence, the hormonal levels of FSH, LH, prolactin and testosterone are restored,
65 entering the testis of the Syrian hamster spontaneously in recrudescence (Matt &
66 Stetson, 1979; Turek et al., 1975).

67 These stages of regression and testicular recrudescence marked by seasonal
68 reproduction are reflected in cellular alterations in the seminiferous epithelium, which
69 depend on the precise regulation between two opposing activities - proliferation and
70 apoptosis (Pastor et al., 2011). Several authors have discussed the possible cellular
71 mechanisms and their degree of involvement in the depletion of the seminiferous

epithelium during testicular regression in various experimental animal models. Some point to cellular desquamation as the main mechanism of seminiferous epithelial atrophy (Dadhich et al, 2010, Dadhich et al., 2013); others point to the programmed death of germ cells in mammals (Young et al., 1999,Young et al. 2001c) accompanied, in the case of birds, by Sertoli cell apoptosis (Young et al., 2001b). For other authors, the cause of reduced sperm formation in the non-breeding season is the reduction of spermatogonia proliferation (Roelants et al., 2002). Recent studies in the Syrian hamster indicate that increased germ cell and Sertoli cell apoptosis, decreased spermatogonial proliferation and the phagocytosis of late spermatids would be major causes of depletion of the seminiferous epithelium in the regression following a short photoperiod (Seco-Rovira et al., 2014, 2015).

When recrudescence is initiated, the testis gradually regains its size and weight, returning to complete spermatogenesis with normal spermiogenesis. All this is a consequence of the restoration of the hormonal levels of FSH, LH and testosterone. In the Syrian hamster, the increase in plasma FSH may act as a stimulus for germ cell proliferation, which guides the reestablishment of the spermatogenic process. This is determined by an increase in PCNA positive cells, which correlates with the recovery of spermatogenesis (Jin et al., 2002). In regards to apoptosis in the seminiferous epithelium of rodents during recrudescence, a decrease in the same has been observed in the white-footed mouse (Young et al., 2001a). In the Syrian hamster, low temperatures and hibernation may produce a delay in recrudescence due to increased apoptosis but without altering the proliferation of spermatogonia (Sato et al., 2005). Finally, although the histomorphometry of regression has recently been described in the Syrian hamster (Seco-Rovira et al., 2015), little is known about changes in the seminiferous epithelium in Syrian hamsters undergoing recrudescence as compared with the regressed state,

97 other than the observed changes in testicular, interstitial and blood vessel volumes
98 (Russell et al., 1994).

99 Taking into account all the above and in order to characterize the process of
100 recrudescence both histologically and at cellular level, the following objectives were
101 proposed: a) to determine the role of proliferation and apoptosis phenomena in the
102 recovery of the seminiferous epithelium in Syrian hamster (*Mesocricetus auratus*)
103 during spontaneous recrudescence after exposure to short photoperiod, and b) to
104 histomorphometrically characterize the testis, with particular regard to its tubular
105 compartment.
106

2. MATERIAL AND METHODS

2.1 Animals and sampling

For the experiment, 25 sexually mature 6-month-old male hamsters (*Mesocricetus auratus*) were maintained initially with a 14:10 h light-dark photoperiod. Five healthy and sexually mature 6-month-old male hamsters (*Mesocricetus auratus*) were used as Control group and maintained with the same 14:10 h light-dark photoperiod. The animals were kept in the animal facility of the University of Murcia at a room temperature of 20 °C and provided with food and water *ad libitum*. In addition, twenty 6-month-old animals were subjected to a short photoperiod of 8:16 h light-dark. At 16, 19 and 21 weeks, males exposed to short photoperiod plus controls were killed by overdose of CO_2 in a closed chamber and the testes were immediately removed. The study was performed according to the ethical and legal standards mentioned in Spanish Royal Decree RDL 53/2013 of 10th October concerning the protection of animals used for experimentation and other scientific proposes, and 2010/63/EU legislation on animal protection. Part of the testis was fixed in Methacarn solution (Chloroform, Methanol and Acetic Acid 6:3:1) for 8 h at room temperature before being processed and embedded in paraffin. Three recrudescence groups were established: initial recrudescence (IR), advance recrudescence (AR) and total recrudescence (TR). These groups were established according to the time of exposure to the short photoperiod (IR: 16 weeks; AR: 19 weeks; TR: 21 weeks). The proportion of tubular sections showing spermatogenesis recovery (used as internal control of the homogeneity of the groups) was: normal spermatogenesis category (IR: 0% ± 0; AR: 14.3% ± 2.6; TR: 21.3% ± 2.4; Control: 44.8% ± 3.4), testicular volume (10^3) (IR: 0.59± 0.07 mm^3; AR: 1.44 ± 0.046 mm^3; TR: 1.73 ± 0.06 mm^3; Control: 2.06 ± 0.11 mm^3) and weight (g) (IR: 0.61± 0.08;

AR: 1.49 ± 0.04; TR: 1.79 ± 0.06; Control: 2.1 ± 0.11) (Martínez-Hernández et al. 2015).

2.2 Proliferating Cell Nuclear Antigen (PCNA) immunohistochemistry and TUNEL labelling assay

The samples were deparaffinized with xylene, rehydrated in ethanol (100%, 96% and 70%) and distilled water. The peroxidase activity was quenched with 0.3% H_2O_2 (30 minutes) at room temperature. In the immunohistochemistry technique, the samples were incubated in a humidity chamber with the PCNA antibody (Biomeda, Foster City, CA; USA) diluted 1:200 overnight at 4 ºC, washed with PBS and incubated with biotinylated Polyclonal Rabbit anti-mouse immunoglobulin (Dako, Glostrup, Denmark) diluted 1:200 for 45 minutes. After washing with PBS, samples were incubated with Streptavidin conjugated with HRP (Horseradish Peroxidase) (Dako, Glostrup, Denmark) diluted 1:300 for 30 minutes and revealed the peroxidase activity with a substrate–chromogen solution containing 0.025 mg/ml 3, 3´ diaminobenzidine (DAB (Sigma Chemical Co.)) and 0.015 % hydrogen peroxide (Seco-Rovira et al., 2015).

In the histochemistry technique, the TUNEL reaction Kit was used according to the protocol of the "Cardio TACS® *In Situ* Apoptosis Detection Kit" (R&D Systems, Minneapolis, USA). The samples were immersed in 1X TdT labelling buffer (1M TACS Safe-TdT Buffer, 0.5 mg/ml BSA, 0.6 mM 2-mercaptoethanesulfonic acid) at 18-24 ºC for 5 minutes and incubated for one hour at 37 ºC with TdT dNTP Mix (0.25 mM biotinylated dNTP), 50x Mn^{+2} (1µl), TdT Enzyme (1µl) and 1X TdT labelling buffer. The reaction was stopped with TdT stop buffer (0.1 M EDTA, pH 8.0) and incubated with streptavidin-horseradish peroxidase for 10 minutes at 18-24 ºC, stained with TACS

Blue Label and incubated with Contrast C solution (Nuclear Fast Red) (Seco-Rovira et al., 2015).

2.3 Qualitative and semiquantitative histological study

For the semiquantitative study of the degree of tubular recovery, three randomly chosen 5 μm-thick sections of each testis, were used and 25 tubular cross-sections were examined from each section using the Cell D Olympus image analysis software. The degree of tubular seminiferous recovery was classified into 4 categories: a) Normal spermatogenesis, when the tubular epithelium contained the entire series of well-differentiated germ cells; b) hypospermatogenic, when a reduction in late spermatid development and thinning of the germinal epithelium were evident; c) round spermatids in apparent arrest, when the germinal epithelium had developed until (but not beyond) the appearance of round spermatids (no late or elongated spermatids); d) spermatocytes in apparent arrest, when the germinal epithelium had developed only until spermatocytes were observed and no (or few) round spermatids could be observed. The mean tubular diameter (MTD) was calculated using the Cell D Olympus analysis software according to a previous study (Seco-Rovira et al., 2015).

2.4 Quantitative histomorphometric study

The testes were weighed immediately after removal and the testicular volumes ($\mathbf{V_T}$) were estimated by considering a testis density (ρ) of 1.037 g/cm^3 (Sinha Hikim et al., 1988, Seco-Rovira et al., 2015) and the testis weight (T_W) ($\mathbf{V_T = T_W / \rho}$).

Three 5 □m sections (25 random fields per section) were chosen and the following histomorphometric variables were calculated using the Cell D Olympus image analysis software: (1) the volume density of seminiferous tubules ($\mathbf{VD_{ST}}$), as the seminiferous

tubule surface/reference area (142,049.28 μm^2) ratio; (2) the testicular interstitium volume density (**VD_{TI}**), as the ratio of interstitium surface/reference area (142,049.28 μm^2) ratio; (3) the total volume of seminiferous tubule per testis (**V_{ST}**) multiplying $VD_{ST} \times V_T$; (4) the length of seminiferous tubule per volume unit (**L_v**) using the established formula: $L_v = VD_{ST}/\pi R^2$ (Santamaria et al., 1995). In addition, for the total length of seminiferous tubules (**L_{ST}**), all tubules were assumed to form a single cylinder with a length L, a radius R (MTD/2) and a volume V_{ST}. Then, L_{ST} was calculated as $L_v \times V_T$; (5) the total tubular area **(TTA),** as $2\pi R \times L_{ST}$; (6) the total volume of the seminiferous epithelium (**VD_{SE}**) was calculated from the volume density of seminiferous epithelium (seminiferous epithelium surface/reference area (142,049.28 μm^2) ratio) multiplying by **V_T**; (7) the volume of the testicular interstitium **(VD_{TI})** was calculated from the volume density of testicular interstitium (testicular interstitium surface/reference area (142,049.28 μm^2) ratio) multiplying by **V_T**; (8) the volume of the tubular lumen (**VD_{TL}**) as tubular lumen surface/reference area (142,049.28 μm^2) ratio multiplying by **V_T**. Finally, the **VD_{SE}/VD_{TL}** ratio was calculated, and a Pearson correlation study between all the histomorphometric variables studied was performed.

2.5 Semiquantitative evaluation of the immunohistochemical and TUNEL study

The methodology for the proliferation index (PI) was determined by counting the number of positive spermatogonia x 100/total spermatogonia (positive plus negative) as in a previous study (Seco-Rovira et al., 2015). The apoptotic activity of the seminiferous epithelium during recrudescence after exposure to a short photoperiod was assessed using TUNEL, following the methodology used in a previous study (Seco-Rovira et al., 2015). The apoptotic index (AI) of each cell type was calculated as the number of TUNEL + germ cell types x100/number of total germ cell type (positive plus

negative). The apoptotic indexes (AI) and their relationship with PI were calculated according to a previous study (Seco-Rovira et al., 2015).

2.6 Quantitative study of proliferative and apoptotic activities and transmission electron microscopy (TEM) study

The numerical density of germ cells **(Nd)** was calculated using the Floderus equation (Floderus S, 1944; Morales et al., 2003; Seco-Rovira et al., 2015). The total number of germ cells were obtained by multiplying **Vd** (obtained with the Floderus equation) and **VT**. The ultrastructural study was realized according to a previous study (Seco-Rovira et al., 2015).

2.7 Statistical analysis

A one way analysis of variance was applied followed by a *post hoc* test contrasting equality between pairs of means, using least significance difference (LSD). Some data were logarithmically transformed for statistical analysis. Also, a Pearson's correlations analysis was performed with the data obtained. The results were considered statistically significant when the P-value was less than 0.05. The results are presented as average ± standard error of the mean in the text and tables. The SPSS 19 statistical software package supplied by the University of Murcia under license was used.

3. RESULTS

3.1 Histomorphometric study of the testicular recrudescence in Syrian hamster after short photoperiod

The histology of the seminiferous tubules of the established groups differed as regards the type of tubular section (different degrees of epithelial recovery and normal spermatogenesis). In the Control group, most sections showed normal spermatogenesis or were hypospermatogenic. For the IR group, tubular sections in apparent spermatid arrest were the most frequent, and no normal spermatogenesis tubular sections were found. In the AR group, a sharp increase in hypospermatogenic tubular sections was observed, while there was a low proportion of normal tubular sections and those in "apparent" spermatid arrest. Finally, the TR group seemed to be in an intermediary stage between AR and the control group (Fig.1 and 2). When the mean tubular diameter (**MTD**) in IR was compared to the AR and TR diameters, the diameter was seen to increase significantly as spontaneous recrudescence progressed (Table 1). As regards testicular weight, a significant increase in all the established groups was observed, with significant increases in $\mathbf{V_T}$, $\mathbf{V_{ST}}$ and $\mathbf{V_{SE}}$. $\mathbf{V_{TI}}$ was significantly lower in the IR group than in the other groups, but without significant differences. The $\mathbf{V_{TL}}$ and **TTA** were significantly lower in IR than in the other groups and were always lower than in the Control group. $\mathbf{L_{ST}}$ was significantly lower in the IR group than in the other groups, without significant differences. Finally, the $\mathbf{VD_{SE}}$ / $\mathbf{VD_{TL}}$ ratio was only significantly higher in the IR group compared with the rest of the groups. All the histomorphometric variables studied showed a positive correlation with respect to testicular volume and weight ($p<0.05$). In addition, $\mathbf{V_{ST}}$ positively correlated ($p<0.05$) with all the variables studied ($p<0.05$), except $\mathbf{V_{TI}}$. However, no correlations between $\mathbf{V_{TI}}$ and $\mathbf{V_{SE}}$, $\mathbf{V_{TI}}$ and

MTD were found. Finally, a positive correlation between **L_{ST}** and **V_{TI}** but no correlation between **L_{ST}** and **MTD** were observed.

3.2 Proliferation and apoptosis in the seminiferous epithelium during testicular recrudescence after exposure to short photoperiod.

Proliferating spermatogonia were identified by PCNA, and the TUNEL technique allowed identification of apoptotic germ cells (Fig. 3 and 4). The proliferation index (PI) of the spermatogonia was significantly higher in the three recrudescence groups (IR, AR and TR) than in the Control group, with no significant differences among the recrudescence groups (Table 3). By contrast, the apoptotic index (AI) returned to similar values to those of the Control group during recrudescence. Thus, the AI of spermatogonia was significantly higher in the IR group than in the TR and Control groups. The AI of spermatocytes and round spermatids were significantly higher in the IR group than the other groups (Table 3). With the proliferation and apoptosis results obtained above, the relationship between the two phenomena during testicular recrudescence was studied (Table 3).

The total number of spermatogonia and spermatocytes increased significantly from IR to AR, while the total number of round spermatids was significantly lower in IR than in the other groups, which did not reach the values of the Control group (Table 4).

With TEM in early recrudesce, spermatocytes and spermatids in apoptosis were observed in medial position and next to the lumen. In addition, the complete loss of adhesion with neighbouring cells, chromatin condensation and a degenerated cytoplasm, typical of programmed death were observed. Finally, both spermatogonia in division (metaphase and anaphase) and some abnormalities which affected the structure of the acrosome (abnormal acrosome formation and abnormal condensation of chromatin) were evident, especially in the IR group, (Fig. 5).

4. DISCUSSION

Few studies have described the sequence of histological changes that occur in the seminiferous tubule during the recrudescence process (Schlatt et al., 1995, Young et al., 2001a). Our study in the Syrian hamster provides new insights into how the recovery of the seminiferous epithelium takes place during recrudescence. At the beginning of recrudescence (IR), most of the seminiferous tubules begin to recover, while parts of them are in a state similar to total regression. Although parts of the seminiferous tubules are in spermatid arrest in the AR group, a very important change takes place since most of the tubules show almost complete spermatogenesis, while other tubule parts appear to be in a normal state. This suggests that the tubular epithelium is in a transitional situation since its recovery is not yet homogeneous, as demonstrated by both the heterogeneity of the sections found and the transition between the percentages of tubular sections in each state during recrudescence. Finally, in the TR group, the seminiferous epithelium has almost fully recovered to reach Control levels.

Several authors have described an increase in seminiferous tubule diameter during recrudescence in various animal species. In the Djungarian hamster, for example, this increase is due initially to the increase of primary spermatocytes followed by the appearance of spermatids (Schlatt et al., 1995). In the Syrian hamster, changes in tubular diameter were studied during the recrudescence and compared to animals with completely regressed or active testis (Jin et al., 2002). Their results are similar to ours since there was an increase of tubular diameter throughout the process of recrudescence until a similar diameter to that observed in the control group was reached. In our study, the diameter underwent a significant increase between IR and AR, coinciding with the increase observed in the proportion of sections of the seminiferous tubules that already showed spermiogenesis, suggesting an increase in the number of cells.

Electron microscopy indicated that during the restoration of spermiogenesis aberrations occur that affect the acrosome particularly. Such aberrations are also observed in the adult Syrian hamster, where they increase with age (Calvo et al., 1995, Morales et al., 2004), and probably generate non-competent spermatozoa to fertilize. In fact, in hamsters, the full fertilisation capacity of the spermatozoa obtained from the tail of the epididymis is not reached until nine weeks after the animals have been exposed to a long photoperiod after the testicular regression that takes place during a short photoperiod (Holland et al., 1987).

The proliferative activity of spermatogonia during recrudescence was superior to the degree of proliferation found in the Control group. In a previous study, which did not provide quantitative data, an increase in the number of PCNA+ cells was observed during induced recrudescence, and was dependent on the progression of this process (Jin et al., 2002). In our study, it was observed that both the number of total spermatogonia and proliferative spermatogonia increased for a significant period of time until they stabilized in the AR group. Such stabilization coincides with the apoptotic/proliferative activity ratio in this group, which was similar to that of the control animals. In this way, it is possible to affirm that the epithelium has already reached a balance between the generation of new germ cells and their apoptosis, reflecting the equilibrium that exists during long photoperiod.

As regards apoptosis in recrudescence, the results obtained show that at the beginning of the recrudescence process, apoptotic activity still affected the seminiferous epithelium, although to a lesser extent than during total regression (Seco-Rovira et al., 2015). Thus, in the AR group, apoptosis returns to values similar to those of the controls, facilitating the recovery of spermatogenesis and the appearance of a greater number of tubular sections in hypoespermatogenesis. The rate of apoptosis of

317 spermatogonia only remains slightly higher in AR, which is to be expected given the
318 fact that the rate of proliferation, which was high during total regression (Seco-Rovira et
319 al., 2015), is maintained in the AR group above control group levels. In summary,
320 considering the overall recrudescence process, apoptosis would act as a brake on
321 spermatogenesis during the initial process, and cease to act half way through the
322 process. Other experimental studies with the white-footed mouse identified a similar
323 phenomenon during recrudescence. In this seasonally reproducing species which shows
324 testicular regression in short photoperiod, it was also observed that the apoptotic activity
325 decreases from the time of total regression (significantly so in the first few weeks of
326 recrudescence) and later varies very little from the long photoperiod controls (Young et
327 al., 2001a). The authors concluded by indicating that the decrease in apoptosis occurs
328 prior to the activity of the testis and that the recrudescence would have two phases - an
329 acute initial phase with the cessation of apoptosis followed by a period of cell
330 proliferation and differentiation. Our results in the Syrian hamster, where
331 proliferation/apoptosis were studied together, show that, along with the decrease in
332 apoptotic phenomena during the first part of recrudescence, an increase in proliferative
333 activity occurs from the beginning of recrudescence, meaning that the cellular
334 mechanism of recovery of the seminiferous epithelium would be slightly different in
335 this species. There would also be two phases in the recovery of the seminiferous
336 epithelium - a first phase when proliferation would be acting alongside the decrease in
337 apoptosis, and a second one where cellular proliferation and differentiation would
338 predominate.

339 The numbers of both spermatogonia and spermatocytes increased compared to the
340 values observed in total regression (Seco-Rovira et al. 2015) and these cell types
341 returned to similar levels to those of the controls towards the middle of the

342 recrudescence process. In the case of the spermatids, their number increased gradually
343 without reaching control values. These facts indicate that in our TR group the balance
344 that exists in the Control group concerning the process of meiosis and formation of
345 spermatids, which later differentiate into spermatozoa, has still not been reached in the
346 TR group. As is well known, this balance is highly dependent on the apoptosis of
347 spermatocytes (Sinha Hikim, 1999). In this sense, the lowest rate of apoptosis of
348 spermatocytes was observed in the TR group, enabling a greater number of them to
349 produce more spermatids, probably reaching control values at a time after the limit
350 imposed in this study.

351 The changes found in relation to the increase in testicular weight and volumes
352 during recrudescence are commonly observed in other species and indirectly indicate a
353 restoration of spermatogenesis (Schlatt et al., 1995, Young et al., 2001a). In addition to
354 the other histomorphometric parameters analysed in this work, there are studies about
355 the changes that occur in tubular, epithelial and interstitial volumes and seminiferous
356 tubule length, but only comparing fully regressed testes of animals with those of
357 sexually active animals (Sinha-Hikim et al., 1988). From the data obtained in the
358 present study, it should be noted first that all variables were always higher in the IR
359 group than during total regression (Seco-Rovira et al., 2015), which indicates that
360 recrudescence has already begun in the first group. Second, the strong increase of the
361 tubular volume between IR and AR seems to depend on the significant increase in
362 tubular length and the tubular diameter. Indeed, the results seem to indicate that at the
363 beginning of recrudescence the increase in the volume of the seminiferous tubule is due
364 to the increase in both its width and its length. In the AR group, this length reaches a
365 value close to that found in the Control group. This behaviour of tubule length implies
366 an increase in interstitial volume, which also occurs before reaching its final size in AR.

Third, another phenomenon involves a moderate increase in tubular volume between AR and TR, when values are restored to reach values similar to those of the control group. This growth in volume seems to depend mainly on the width of the tubule. Therefore the results obtained suggest that recovery during recrudescence is biphasic, whereby the increase in tubular volume first depends on the increase of its length followed by another stage when the increase in tubular volume depends more on the increase in width.

In conclusion, the spontaneous recrudescence observed after exposure to short photoperiod showed an epithelial recovery that is a consequence of both a decrease in apoptosis in the first half of the process and a sustained increase in the proliferative activity of spermatogonia and their subsequent differentiation into spermatocytes during recrudescence. This process involves the restoration of the tissue structure of the testis, which manifests itself first as an increase in tubule length accompanied by restoration of the volume of the interstitium, with a gradual increase in tubular diameter. This is in line with the gradual and sequential recovery of both the tubular and epithelial volumes and the percentage of tubular sections showing complete spermatogenesis.

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AUTHOR CONTRIBUTIONS

Jesús Martínez Hernández was involved in the design of the paper, image and data acquisition, analysis, interpretation of the literature and drafting the paper. Vicente Seco-Rovira, Ester Beltran-Frutos, Concepción Ferrer and Maria del Mar Sanchez-Huertas participated in the analysis and interpretation of the literature. Manuel Canteras participated in the statistical analysis of the data. Luis M. Pastor participated in all phases of the paper's elaboration. All authors participated in the revision and corrections of the manuscript and approved the final manuscript. Neither author has any conflict of interest to declare.

400 **REFERENCES**

401 Berndtson, W.E. & Desjardins, C. (1974). Circulating LH and FSH levels and
402 testicular function in hamsters during light deprivation and subsequent photoperiodic
403 stimulation. *Endocrinology,* 95, 195-205.

404 Bronson F.H. (1999). Puberty and energy resources: A walk on the wild side. In:
405 Reproduction in Context, (eds K. Wallen & J.S Schnider), pp 15-33, MIT Pres,
406 Cambridge, MA

407 Calvo, A., Pastor, L.M., Gallego-Huidobro, J., Horn, R., Pallares, J. (1995).
408 Abnormal spermatozoa in the cauda epididymidis of adult and aged hamsters
409 (*Mesocricetus auratus*): a study by electron microscopy. *Acta Anatomica (Basel),* 154,
410 186-95.

411 Dadhich, R.K., Barrionuevo, F.J., Real, F. M., Lupiañez, D.G., Ortega, E, Burgos,
412 M & Jiménez, R. (2013). Identification of live germ-cell desquamation as a major
413 mechanism of seasonal testis regression in mammals: a study in the Iberian mole (*Talpa*
414 *occidentalis*). *Biology of Reproduction,* 88, 101.

415 Dadhich, R.K., Real, F.M., Zurita, F., Barrionuevo, F.J., Burgos, M. & Jiménez,
416 R. (2010). Role of apoptosis and cell proliferation in the testicular dynamics of seasonal
417 breeding mammals: a study in the Iberian mole, *Talpa occidentalis*. *Biology of*
418 *Reproduction,* 83, 83-91.

419 Floderus, S. (1944). Untersuchungen uber den Bauder menschlichen Hypophyse
420 mit besonderer. Beruchsichtigung der quantitativen mikromorphologischen verhaltnisse.
421 *Acta Pathologica Microbiolgica Scandinava,* 53, 1–276.

422 Hikim, A.P., Bartke, A.J. & Russell L.D. (1988). The seasonal breeding hamster
423 as a model to study structure-function relationships in the testis. *Tissue and Cell*, 20, 63-
424 78.

425 Holland, M.K., Rogers, B.J., Orgebin-Crist, M.C. & Danzo, B.J. (1987). Effects
426 of photoperiod on androgen-binding protein and sperm fertilizing ability in the hamster.
427 *Journal Reproduction Fertility*, 81, 99-112

428 Jin, W., Herath, C.B., Yoshida, M., Arai, K.Y., Saita, E., Zhanquan, S., Taya, K.
429 (2002). Inhibin B regulating follicle-stimulating hormone secretion during testicular

recrudescence in the male golden hamster. *Journal of Andrology,* 23, 845-853.

Kelly, K.K., Goldman, B.D. & Zucker, I. (1994). Gonadal growth and hormone concentrations in photoregressed Siberian hamsters: pinealectomy versus photostimulation. *Biology and Reproduction*, 51, 1046-1050.

Matt KS & Stetson MH. (1979) Hypothalamic-pituitary-gonadal interactions during spontaneous testicular recrudescence in golden hamsters. *Biology of Reproduction* 20, 739-746.

Martínez-Hernández, J., Seco-Rovira, V., Beltran-Frutos, E., Ferrer, C., Quesada-Cubo, V., Canteras, M. & Pastor, L.M. (2015). Proliferative and apoptotic activity changes in seminiferous epithelium of Syrian hamster (*Mesocricetus auratus*) during recrudescence after to short photoperiod. *Histology and Histopathology,* 30 (Supplement 1), 3.

Morales, E., Pastor. L. M., Horn, R., Zuasti, A., Ferrer, C., Calvo, A., Santamaría, L. & Canteras, M. (2003). Effect of ageing on the proliferation and apoptosis of testicular germ cells in the Syrian hamster Mesocricetus auratus. *Reproduction Fertility and Development* 15, 89-98.

Morales, E., Horn, R., Pastor, L.M., Santamaría, L., Pallarés, J., Zuasti, A., Ferrer, C., Canteras, M. (2004). Involution of seminiferous tubules in aged hamsters: an ultrastructural, immunohistochemical and quantitative morphological study. *Histology and Histopathology,* 19, 445-55.

Pastor, L.M., Zuasti, A., Ferrer, C., Bernal-Mañas, C.M., Morales, E., Beltrán-Frutos, E. & Seco-Rovira. V. (2011). Proliferation and apoptosis in aged and photoregressed mammalian seminiferous epithelium, with particular attention to rodents and humans. *Reproduction in Domestic Animals,* 46, 155-164.

Roelants, H., Schneider, F., Göritz, F., Streich, J. & Blottner S. (2002). Seasonal changes of spermatogonial proliferation in roe deer, demonstrated by flow cytometric analysis of c-kit receptor, in relation to follicle-stimulating hormone, luteinizing hormone, and testosterone. *Biology of Reproduction,* 66, 305-312.

Russell, L.D., Chandrashekar, V., Bartke, A. & Hikim A.P. (1994). The hamster Sertoli cell in early testicular regression and early recrudescence: a stereological and endocrine study. *International Journal of Andrology,* 17, 93-106.

Santamaría L, Martín R, Codesal J & Paniagua R. (1995) Myoid cell proliferation in rat seminiferous tubules after ischaemic testicular atrophy induced by epinephrine. Morphometric and immunohistochemical (bromo-deoxyuridine and PCNA) studies. *Int J Androl* 18, 13-22.

Sato, T., Tachiwana, T., Takata K., Tay, T.W., Ishii, M., Nakamura, R., Kimura, S... Hayashi, Y. (2005). Testicular dynamics in Syrian hamsters exposed to both short photoperiod and low ambient temperature. *Anatomia Histologia Embryologia,* 34, 220-224.

Schlatt, S., De Geyter, M., Kliesch, S., Nieschlag, E. & Bergmann M. (1995). Spontaneous recrudescence of spermatogenesis in the photoinhibited male Djungarian hamster, *Phodopus sungorus*. *Biology of Reproduction,* 53, 1169-1177.

Seco-Rovira, V., Beltrán-Frutos, E., Ferrer, C., Saez, F.J., Madrid, J.F., Canteras, M. & Pastor, L.M. (2015). Testicular histomorphometry and the proliferative and apoptotic activities of the seminiferous epithelium in Syrian hamster (*Mesocricetus auratus*) during regression owing to short photoperiod. *Andrology,* 3, 598-610.

Seco-Rovira, V., Beltrán-Frutos, E., Ferrer, C., Sáez, F.J., Madrid, J.F. & Pastor, L.M. (2014). The death of sertoli cells and the capacity to phagocytize elongated spermatids during testicular regression due to short photoperiod in Syrian hamster (*Mesocricetus auratus*). *Biology of Reproduction,* 90, 107.

Sinha Hikim, A.P., Bartke, A. & Russell, L.D. (1988). Morphometric studies on hamster testes in gonadally active and inactive states: light microscope findings. *Biology of Reproduction,* 39, 1225-1237.

Sinha Hikim, A.P. & Swerdloff, R.S. (1999). Hormonal and genetic control of germ cell apoptosis in the testis. *Review of Reproduction* 4, 38-47.

Turek, F.W., Elliott, J.A., Alvis, J.D. & Menaker, M. (1975). Effect of prolonged exposure to nonstimulatory photoperiods on the activity of the neuroendocrine-testicular axis of golden hamsters. *Biology of Reproduction,* 13, 475-481.

Young, K. A., Zirkin, B. R. & Nelson, R. J. (1999). Short photoperiods evoke testicular apoptosis in white-footed mice (*Peromyscus leucopus*). *Endocrinology* **140**, 3133-3139.

Young, K.A., Zirkin, B.R. & Nelson, R.J. (2001a). Testicular apoptosis is down-

492 regulated during spontaneous recrudescence in white-footed mice (*Peromyscus*
493 *leucopus*). *Journal of Biological Rhythms* 16, 479-488.

494 Young, K.A., Ball, G.F. & Nelson, R.J. (2001b). Photoperiod-induced testicular
495 apoptosis in European starlings (*Sturnus vulgaris*). *Biology of Reproduction,* 64, 706-
496 713.

497 Young, K.A. & Nelson, R.J. (2001c). Mediation of seasonal testicular regression
498 by apoptosis. *Reproduction,* 122, 677-685.

499

500

501 **Table 1.** Semiquantitative data of Mean Tubular Diameter (MTD) from testicular recrudescence

502 groups after short photoperiod.

Stage/Phase	Initial Recrudescence	Advanced Recrudescence	Total Recrudescence	Control
Number of Animals	5	8	7	5
MTD (μm)	178.91 ± 12.73^{a}	244.68 ± 9.02^{b}	272.62 ± 11.36^{b}	283.60 ± 19.18^{b}

503 $P<0.05$. Data shown as mean ± SEM

504

505 **Table 2.** Quantitative data obtained in groups of testicular recrudescence due to short

506 photoperiod for the various parameters studied.

	Initial Recrudescence	Advanced Recrudescence	Total Recrudescence	Control
Testis Weight (g)	0.61 ± 0.08^{a}	1.49 ± 0.04^{b}	1.79 ± 0.06^{c}	2.14 ± 0.14^{d}
$*V_T$ $(10^3)(mm^3)$	0.59 ± 0.07^{a}	1.44 ± 0.04^{b}	1.73 ± 0.06^{c}	2.06 ± 0.14^{c}
$*V_{ST}$ $(10^3)(mm^3)$	0.37 ± 0.06^{a}	1.03 ± 0.06^{b}	1.33 ± 0.06^{c}	1.48 ± 0.13^{c}
$*V_{SE}$ $(10^3)(mm^3)$	0.32 ± 0.05^{a}	0.82 ± 0.04^{b}	1.07 ± 0.04^{c}	1.18 ± 0.08^{c}
$*V_{TL}$ $(10^2)(mm^3)$	0.52 ± 0.05^{a}	1.93 ± 0.27^{b}	$2.37 \pm 0.17^{b,c}$	2.73 ± 0.20^{c}
$*V_{TI}$ $(10^2)(mm^3)$	2.11 ± 0.23^{a}	4.04 ± 0.40^{b}	3.99 ± 0.51^{b}	4.80 ± 1.09^{b}
$*TTA(10^3)(mm^2)$	8.30 ± 0.84^{a}	16.81 ± 0.65^{b}	$19.60 \pm 0.90^{b,c}$	20.86 ± 0.88^{c}
$*L_{ST}$ (10^3) (mm)	14.73 ± 0.94^{a}	22.01 ± 1.11^{b}	22.32 ± 1.71^{b}	22.36 ± 1.48^{b}
Relationship V_{SE}/ V_{TL}	6.15 ± 0.52^{a}	4.64 ± 0.46^{b}	4.61 ± 0.29^{b}	4.32 ± 0.34^{b}

507 $P<0.05$. Data shown as mean ± SEM.

508 * Statistics made with ln-transformed data.

509 $\mathbf{V_T}$: Testicular Volume. $\mathbf{V_{ST}}$: Volume of Seminiferous Tubule. $\mathbf{V_{SE}}$: Volume of Seminiferous

510 Epithelium. $\mathbf{V_{TL}}$: Volume of Tubular Lumen. $\mathbf{V_{TI}}$: Volume of Testicular Interstitium. **TTA**: Total Tubular

511 Area $\mathbf{L_{ST}}$: Length of Seminiferous Tubule

512

513 **Table 3.** Semiquantitative data for proliferation and apoptosis in the different testicular

514 recrudescence groups.

	Initial Recrudescence	Advanced Recrudescence	Total Recrudescence	Control
Proliferation Index **(PI)**	94.57 ± 0.46[a]	98.05 ± 0.48[a]	95.59 ± 1.28[a]	81.79 ± 3.07[b]
Apoptotic Index **(AI)**				
Round spermatids	0.30 ± 0.15[a]	0.04 ± 0.00[b]	0.02 ± 0.00 [b]	0.01 ± 0.00[b]
Spermatocytes	0.88 ±0.25[a]	0.40 ± 0.05[b]	0.17 ± 0.02[b]	0.20 ± 0.02[b]
Spermatogonia	0.50 ± 0.10[a]	0.32 ± 0.08[a,b]	0.11 ± 0.03[b]	0.17 ± 0.01[b]
Spermatogonia + Spermatocytes	0.80 ± 0.21[a]	0.38 ± 0.05[b]	0.16 ± 0.02[b]	0.20 ± 0.01[b]
Total	0.56 ± 0.20[a]	0.19 ± 0.02[b]	0.08 ± 0.01[b]	0.08 ± 0.01[b]
*Relationship (10^{-3})				
AI SG/PI SG	5.34 ± 1.02[a]	3.44 ± 0.90[a,b]	1.36 ± 0.37[b]	2.07 ± 0.22[b]
AI SC/PI SG	51.95 ± 16.42[a]	31.13 ± 5.48[a,b]	14.07 ± 1.90[b]	17.77 ± 2.93[b]
AI SD/PI SG	16.22 ± 5.06[a]	5.70 ± 1.19[b]	4.09 ± 1.30[b b]	2.31±1.06 [b]
AI (SC+SG)/PI SG	57.29 ± 16.58[a]	34.56 ± 5.37[b]	15.43 ± 2.10[b]	19.84 ±3.04 [b]
AI (SG+SC+SD)/PI SG	73.53 ± 20.73[a]	40.27 ± 6.13[b]	19.52 ± 2.79[b]	22.15 ± 3.14[b]

515 P<0.05. Data shown as mean ± SEM.

516 * Statistics made with ln-transformed data.

517 **SG:** Spermatogonia. **SC:** Spermatocytes: **SD:** Round Spermatids.

518

519 **Table 4.** Quantitative data for proliferation and apoptosis in different groups of testicular

520 recrudescence.

	Initial Recrudescence	Advanced Recrudescence	Total Recrudescence	Control
*Total proliferative spermatogonia number per testis (10^6)	17.54 ± 3.72[a]	27.91 ± 2.11[b]	25.72 ± 1.70[b]	27.37 ± 3.39[b]
*Total apoptotic cell number per testis (10^5)				
Round spermatids	2.65± 0.72[a]	1.53 ± 0.31[a]	1.01 ± 0.31[a]	0.75 ± 0.37[a]
Spermatocytes	7.72 ± 1.89[a]	8.11 ± 1.11[a]	3.64 ± 0.53[b]	4.36 ± 0.88[b]
Spermatogonia	0.89 ± 0.22[a]	0.95 ± 0.23[a]	0.35 ± 0.09[a]	0.53 ± 0.24[a]
Spermatogonia + Spermatocytes	8.62 ± 1.94[a]	9.07 ± 1.05[a]	4.00 ± 0.60[b]	4.99 ± 0.90[b]
Total	11.28 ± 2.60[a]	10.58 ± 1.23[a]	5.01 ± 0.71[b]	5.75 ± 1.23[b]
*Total cell number per testis (10^6)				
Round spermatids	111.76 ± 19.79[a]	344.12 ± 11.61[b]	363.37 ± 23.75[b]	428.11 ± 22.60[c]
Spermatocytes	93.11 ± 5.51[a]	198.27 ± 9.37[b]	203.97 ± 9.54[b]	221.71 ± 14.26[b]
Spermatogonia	18.58 ± 3.96[a]	28.46± 2.13 [b]	26.92 ± 1.75[b]	33.13 ± 3.44[b]
Total	223.46 ± 27.96[a]	570.75 ± 18.95[b]	593.21 ± 31.64[b]	675.03 ± 37.75[b]

521 $P<0.05$. Data shown as mean ± SEM

522 * Statistics made with ln-transformed data.

523

524 **FIGURE LEGENDS**

525 **Fig. 1.** Light microscopy. Haematoxylin-eosin staining during testicular
526 recrudescence after exposure to short photoperiod. **(A)** In the IR group, the tubular
527 diameter is reduced and tubular sections, mainly in "apparent" spermatid (empty stars)
528 and spermatocyte (stars) arrest are observed. Detail of a tubular sections in "apparent"
529 spermatid and spermatocyte arrest (Insert). **(B)** In the AR group, increased tubular
530 diameter and number of tubular sections in hypospermatogenesis (star) with a thin
531 epithelium can be observed. Detail of hypospermatogenic tubule (Insert), where a few
532 late spermatids (arrow) can be seen. **(C)** In the TR group, the tubular diameter was
533 similar to the Control and a higher frequency of hypospermatogenic (star) was see.
534 Detail of hypospermatogenic with the epithelium thicker than in previous group (Insert).
535 **(D)** Control group with mainly hypospermatogenic (star) and normal spermatogenesis
536 (empty star) tubular section are seen. Detail of normal tubular section with complete
537 spermatogenesis (Insert). Bars: 100µm. Insert bars: 20 µm. **IR**: initial recrudescence.
538 **AR**: advanced recrudescence. **TR**: total recrudescence.

539

Fig. 2. Variations in the proportions of tubular section types during testicular recrudescence after exposure to short photoperiod: a, and b are statistically significant differences between normal spermatogenesis tubular sections in all study groups when $p<0.05$. a′, b′ and c′ are significant differences between hypospermatogenic tubular sections in all study groups when $p<0.05$. a*, b* and c* are significant differences between spermatid arrest tubular sections in all study groups when $p<0.05$. α, and β are significant differences between spermatocytes arrest tubular sections in all study groups when $p<0.05$

549 **Fig 3.** PCNA immunohistochemistry technique. **(A)** PCNA + cells (arrows) in
550 the IR group in tubular sections in "apparent" spermatid arrest are observed. Some
551 PCNA+ spermatogonia (arrows) and PCNA- spermatogonia (arrowhead) are shown in
552 the insert. In addition, some spermatocytes maintain their reactivity to PCNA. **(B)** In the
553 AR group, PCNA + (arrows) and PCNA- (arrowheads) spermatogonia can be observed.
554 The insert shows a detail of some PCNA+ (arrows) and PCNA- (arrowheads)
555 spermatogonia. **(C)** In the TR group, there is a similar/like proportion (%) of PCNA+
556 spermatogonia (arrows). A detail of some PCNA+ (arrows) and PCNA- (arrowhead) is
557 shown in the insert. **(D)** In the Control group, fewer PCNA+ (arrows) than PCNA-
558 spermatogonia can be observed. The insert shows a detail of some PCNA+ (arrows) and
559 PCNA- (arrowheads) spermatogonia. Bars: 50µm. Insert bars: 25µm. **IR**: initial
560 recrudescence. **AR**: advance recrudescence. **TR**: total recrudescence.

561

562 **Fig 4.** TUNEL histochemistry technique. **(A)** In the IR group, the highest rate of
563 apoptosis for each type of germ cell (spermatogonia (arrow head), spermatocytes,
564 (arrow) and spermatids (empty arrow) was observed. The insert shows a detail of
565 TUNEL+ spermatocytes (arrows) and spermatognia (arrow head) surrounded by other
566 non-apoptotic germ cells. **(B)** In the AR group, a decrease in the number of apoptotic
567 germ cells is observed, although apoptotic spermatocytes, spermatogonia and round
568 spermatids are still observed. The insert shows apoptotic spermatocytes (arrows),
569 spermatogonia (arrow head) and round spermatids (empty arrow) with other healthy
570 germ cells of the seminiferous epithelium. **(C)** In the TR group very low apoptosis of
571 round spermatids in the seminiferous epithelium was observed. The insert shows
572 apoptotic spermatocytes (arrow), spermatogonia (arrow head) and round spermatids
573 (empty arrow). **(D)** In the control group, mainly apoptotic spermatocytes (arrow) are
574 observed. The insert shows some TUNEL+ spermatocytes (arrow) and round spermatid
575 (empty arrow) not commonly seen in this group. Bars: 100 μm. Insert bars: 25μm. **IR**:
576 initial recrudescence. **AR**: advance recrudescence. **TR**: total recrudescence.

577

578 **Fig 5.** TEM of germ cells. A-B. Germ cells in apoptosis. (A) Round spermatid
579 with a typical morphology of the programmed cell death state. (B) Spermatozoa in
580 apoptosis showing separation of the adjacent cells, strong condensation of the chromatin
581 and a cytoplasm in advanced state of degeneration. C-D. Ultrastructure of
582 spermatogonia. (C) Group of type B spermatogonia (arrows) at the base of the
583 seminiferous tubule. (D) Ultrastructure of mitotic spermatogonia, showing the
584 condensation of chromosomes in the metaphase-anaphase. E-F. Abnormalities in
585 spermatogenesis. (E) Round spermatid showing an abnormal acrosome formation
586 (arrow), with an irregular cell nucleus and probably abnormal condensation of the
587 chromatin. (F) Elongated spermatid showing an abnormal acrosome (arrow). Bar: A, C
588 and D= 5μm; B, E and F= 2μm.