Seasonal Variation of Grey Squirrel (Sciurus Carolinesis) Testes is Produced by Mechanisms Distinct from Age Related Testicular Regression

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ABSTRACT

Seasonal breeding is seen in many animal species, and is often associated with a seasonal decrease in testicular size and function. Changes likely occur because sperm and testosterone production are energy-intensive processes which are only feasible when reproductive success is likely. In addition to seasonal changes, testicular regression is a common aging change in many species. It is not clear whether the physiological mechanisms seen are distinct from those in seasonal breeders. Studies reveal anti-aging properties of the gene SIRT1 in the testis, where it prolongs spermatogenesis, suggesting a role in the regulation of testicular size. SOX9, a gene essential for the maintenance of tubular architecture, preventing both feminization and apoptosis of Sertoli cells, is reported to decline with aging. Here we investigate the role of SIRT1 and SOX9 in seasonal testicular regression and maintenance of testicular function, as well as changes in proteins associated with proliferation (PCNA) and apoptosis (Cleaved Caspase 3) in a seasonal breeder. Immunohistochemistry, TUNEL staining and Western Blots were performed on samples of Grey Squirrel (*Sciurus Carolinensis*) testis from different seasons. Winter samples exhibited significantly increased levels of TUNEL (P=0.02) and Cleaved Caspase-3 (P=0.04); these findings indicate higher levels of apoptosis in winter. Changes in PCNA were found to be significant (P=0.002). There was no significant seasonal difference in levels of SIRT1 (P=0.39), suggesting a distinct mechanism from aging related size regression. Interestingly there was significantly higher levels of SOX9 in winter testis (P=0.03), suggesting a role for this gene in seasonal testicular regression.

INTRODUCTION

- Testicular regression in seasonal breeders helps prevent breeding occurring at more energetically costly times of the year.
- Changes seen include a reduction in size, spermatogenesis and therefore decreased fertility. Similar testicular regression is observed with aging.
- It has previously suggested that an increased level of apoptosis is involved in the mechanisms of testicular regression.
- SIRT1 is a gene which encodes for the protein Sirtuin 1, which is known to decrease apoptosis and increase cell survival. Increased expression of Sirtuin 1 leads to a lack of age related testicular regression (Oldknow et al. 2013).
- SOX9 has been found to be essential for adult testis function, a lack of SOX9 causes testicular regression. SOX9 decreases with aging (Mingcai Zhanga et al. 2016) and may contribute to age related testicular regression.

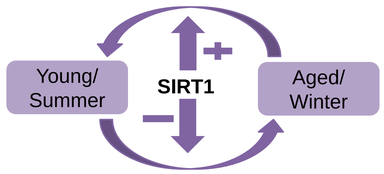

Fig. 1: Diagram depicting aging and seasonal testicular regression. Summer is the breeding season in squirrels and during winter breeding is reduced. Changes seen in these seasons are comparable to that seen in testicular regression. A decrease in SIRT1 is thought to allow age related testicular expression.

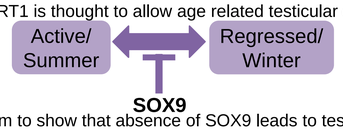

Fig. 2: Diagram to show that absence of SOX9 leads to testicular regression in adult testes. SOX9 is essential for maintenance of Sertoli cell integrity, prevents the loss of seminiferous tubule architecture and feminization of the adult testes, SOX9 also has anti-apoptotic properties (Barrionuevo et al. 2016). A decrease in levels of SOX9 may allow seasonal testicular regression and be involved in age related changes.

AIMS AND OBJECTIVES

Hypothesis:

- Seasonal changes in testis size and function require the activity of pathways involving SIRT1 as seen in testicular regression associated with aging.
- There will be a significant reduction in SOX9 expression in winter testes.
- Higher levels of apoptosis and lower levels of proliferation will be observed in winter

MATERIALS AND METHODS

Obtaining Samples

Grey Squirrels trapped → Maturity status (juvenile/adult) ascertained (Dubock 2009) → Testes collected → Protein lysate obtained and stored at -80 ° C / Fixed with Bouin's fixative and sectioned for IHC

Immunohistochemistry

Dewaxing and Antigen Retrieval → FBS Block → Primary Antibody incubated overnight at 4° C → Secondary Vectastain Elite ABC kit (Vector Laboratories) → Developed with Nova Red kit (Vector Laboratories)

Primary Antibody	Concentration	Secondary Vectastatin ABC
PCNA (Cell Signalling Technology)	1:100	Anti-Mouse
Cleaved Caspase-3 (Abcam)	1:250	Anti-Rabbit
SOX9 (Cell Signalling Technology)	1:100	Anti-Rabbit

Western Blot

Sample prepared and loaded onto gel → Gel electrophoresis at 25mA for 90 minutes → Electroblotting to pure nitrocellulose membrane via trans-blot system at 16V overnight → Primary antibody incubated for 120 minutes at room temperature → Secondary antibody incubated for 60 minutes at room temperature → Detection and imaging of proteins on blots

Primary Antibody	Concentration	Secondary Antibody (1:10,000 concentration)
SIRT1 (Cell Signalling Technology)	1:1000	Anti-Rabbit
PCNA (Cell Signalling Technology)	1:2000	Anti-Mouse
Cleaved Caspase-3 (Abcam)	1:1000	Anti-Rabbit
SOX9 (Cell Signalling Technology)	1:1000	Anti-Rabbit

TUNEL Apoptosis Detection

- Carried out according to ApopTag® Peroxidase Kits instructions (Millipore).

RESULTS

There are significant changes in PCNA expression between the different seasons.

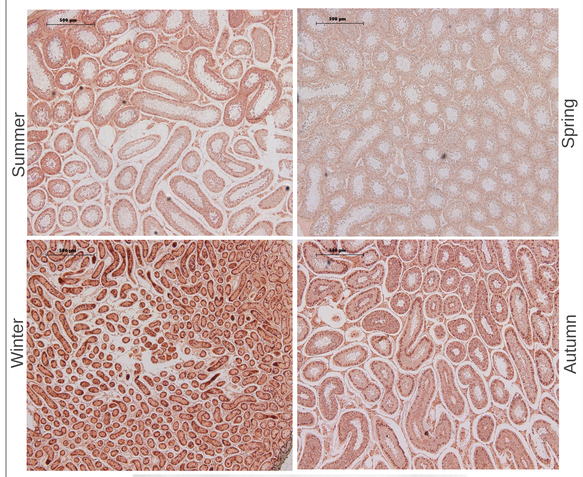

Fig. 3: There is significant seasonal variation of PCNA in the Testis of Grey Squirrels. Immunostaining for PCNA for winter, summer and both intermediate seasons shown above at 5x magnification. Percentage area of cells showing nuclear staining for PCNA is presented in the left hand graph, showing a significant difference (P= 0.002). Western Blot analysis of lysates from grey squirrels is depicted in the right hand graph, there was also significant difference detected in levels of PCNA expressed between the seasons (P=0.0259).

SIRT1 is not involved in seasonal testicular regression.

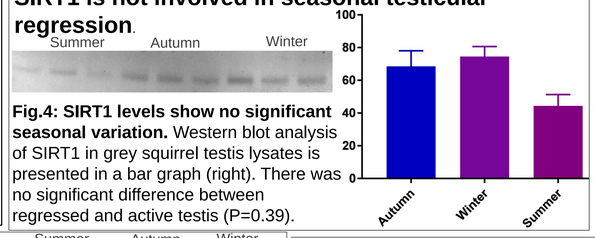

Fig.4: SIRT1 levels show no significant seasonal variation. Western blot analysis of SIRT1 in grey squirrel testis lysates is presented in a bar graph (right). There was no significant difference between regressed and active testis (P=0.39).

Winter grey squirrel testis show significantly higher levels of apoptosis

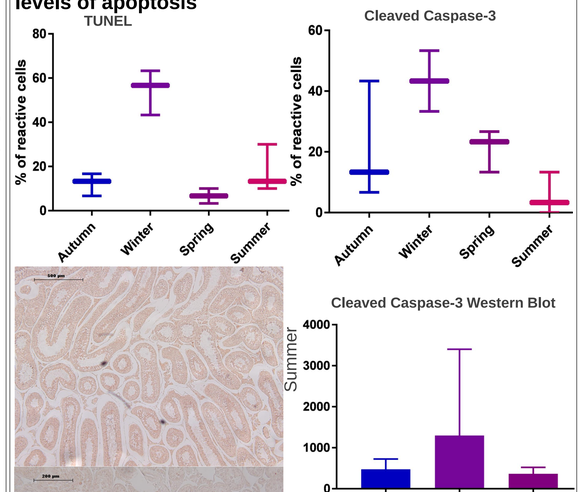

Fig.5: Significantly higher numbers of cells are undergoing apoptosis in winter. Both immunohistochemistry (images to left) and TUNEL showed higher percentage of apoptotic cells in winter (P=0.034 and P=0.013). Western blot analysis found no significant difference in levels of protein found in testis lysate(P=0.72).

SOX9 is dramatically increased in winter Grey Squirrel testis

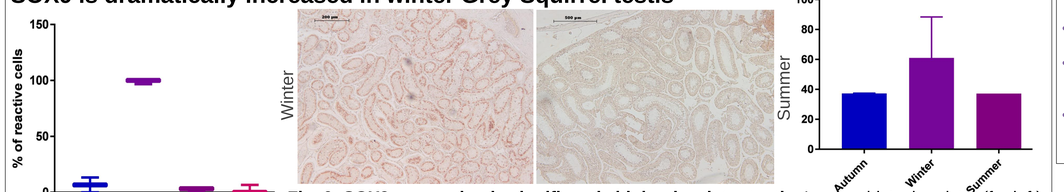

Fig. 6: SOX9 expression is significantly higher in winter testis. Immunohistochemistry (far left) showed a greater percentage of Sertoli cells with nuclear staining for SOX9 in winter (P=0.039). The above images SOX9 nuclear staining at 10x magnification. Western blot analysis (above right) also showed higher levels of SOX9 was found in grey squirrel testis lysate (P=0.039).

CONCLUSIONS

- The pathways of seasonal regression do not involve SIRT1 and are distinct from those of age related regression.
- In winter grey squirrel testes have higher numbers of cells undergoing apoptosis, supporting the theory that increased apoptosis is involved in testicular regression and cessation of spermatogenesis.
- We found increased levels of PCNA in winter grey squirrel testes, suggesting higher levels of early spermatogenesis. The higher levels of apoptosis found may explain why later stages are not observed.
- SOX9 is significantly higher in the winter, it may have a key role in the mechanism of testicular regression and ensuring that seasonal regression is reversible. The permanent changes seen in testicular regression associated with aging may be a result of the loss of SOX9.

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