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BIOMEDICAL SCIENCES

Alterations in the testicular parenchyma of *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ adult mice

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Abstract: Nude mice carry an autosomal recessive mutation in the *Foxn1* gene and therefore are homozygous recessive animals (*Foxn1*$^{-/-}$). The fertility rate of homozygous male (*Foxn1*$^{-/-}$) is low, which seems to be related to the delay in the production of gametes at the beginning of sexual maturity. The present study evaluated the structural and organizational aspects of the testicles of homozygous and heterozygous offspring related to the *Foxn1* gene in mice, describing its implications on spermatogenesis. Adult males Balb/c, *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ mice were used. Testes and epididymis were harvested for histological, biochemical, and sperm transit analyses. Gonadal weight was significantly lower in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals, the same behavior was noticed for the activity of antioxidant enzymes. In addition, tubular parameters such as epithelial proportion, length, and area, as well as germ and Leydig cell's populations were significantly reduced in the aforementioned groups, leading to lower sperm production. In conclusion, our results indicate the importance of the *Foxn1* in Leydig cell's function, reflecting in the preservation of spermatogenesis, thus in germ cell's population and sperm cell production.

Key words: Spermatogenesis, germ cells, Leydig cells, androgen, oxidative stress.

INTRODUCTION

The *Foxn1* gene, located on chromosome 11 (Flanagan 1966), is responsible for encoding the Foxn1 transcription factor, whose expression is found specifically for thymus (Nehls et al. 1996) and skin epithelial cells (Brissette et al. 1996). Loss of function in one or both domains leads to the nude phenotype, commonly used in scientific studies. Nude mice carry an autosomal recessive mutation in the *Foxn1* gene and therefore are homozygous recessive animals (*Foxn1*$^{-/-}$) (Oliveira et al. 2020).

The fertility rate of homozygous females (*Foxn1*$^{-/-}$) is low, which seems to be related to the delay in the production of gametes at the beginning of sexual maturity (Alten & Groscurth 1975) and the impairment of the development of the mammary glands (Nagasawa & Yanai 1977). Thus, the most efficient breeding system uses homozygous males (*Foxn1*$^{-/-}$) and heterozygous females (*Foxn1*$^{+/-}$) (Rygaard & Friis 1974). The homozygous neonate can be identified 24 hours postpartum by the lack or deformity of the vibrissae.

Nude mice have reduced serum concentrations of gonadotropins and testosterone (Rebar et al. 1982), although their synthesis is normal (Brünner et al. 1986). The same pattern can be observed in thymectomized rodents during the neonatal period. These animals have reduced levels of gonadotropins

and testosterone concerning normal ones (Pierpaoli & Besedovsky 1975). Some studies show that Swiss nude mice have an impaired function of the hypothalamic-pituitary-adrenal axis, which could be related to the lack of thymic factors (Daneva et al. 1995). Besides, the thymic factor called thymulin, produced by the thymus epithelial cells shows a physiological role in the communication of the thymus-hypothalamus-pituitary. This was found after the observation that gene therapy, to reestablish the production of thymulin in nude female mice, preventing deficits in the serum concentrations of LH and FSH, typically found in these adult animals (Goya et al. 2007). On the other hand, the concentration of androgens modulates the size of the thymus in rodents, since castrated animals have increased thymus. The evidence of such regulation is the expression of androgen receptors in thymus epithelial cells (Olsen et al. 2001). Therefore, the present study aimed to evaluate the structural and organizational aspects of the testicles of homozygous and heterozygous offspring related to the *Foxn1* gene in mice, describing its implications for the spermatogenic process.

MATERIALS AND METHODS

Animals

The experiment followed the ethics guidelines provided by the National Council for Animal Experimentation Control (CONCEA) and was approved by the Ethics Committee of Animal Use of the Federal University of Viçosa, Brazil (protocol nº 64/2019). All methods agreed with the Brazilian guidelines that involve the use of animals in research (Law 11,794 / 2008).

The fertility rate of homozygous females *Foxn1*$^{-/-}$ is low, as they show a delay in the production of gametes at the beginning of sexual maturity and the development of the mammary glands (Alten & Groscurth 1975, Nagasawa & Yanai 1977). In addition, the lack of fur compromises the heating and well-being of the offspring. For these reasons the most efficient form of reproduction for obtaining *Foxn1*$^{-/-}$ animals is to use homozygous males *Foxn1*$^{-/-}$ and heterozygous females *Foxn1*$^{+/-}$ (Rygaard & Friis 1974). The homozygous neonate can be identified 24 hours postpartum by the lack or deformity of the vibrissae. In the present study mating occurred as previously described, and at 21 days of age 8 homozygous males *Foxn1*$^{-/-}$ and 8 heterozygous males *Foxn1*$^{+/-}$ were separated for the experiment. These animals were compared with homozygous males *Foxn1*$^{+/+}$.

All animals were distributed in individual cages made of opaque polyethylene and closed with stainless steel lids and adequate ventilation. The animals were maintained in controlled photoperiod (12h light/dark cycles) and temperature (21 ºC), with free access to water and chow, being euthanized at 120 days of age.

Thiopental (i.p. 30mg/kg) was used for anesthesia. For each group (*n=8*), the testes and epididymis were removed: six testicles from each group were fixed in Karnovsky fixative (Karnovsky 1965) for histological analysis, while six testicles were stored at -80ºC for oxidative stress analysis. Four testicles with their epididymis were frozen and kept freezer at -20ºC for sperm transit analysis.

Histology

The testes were fixed for 24h. The albuginea was removed, weighed, and its weight subtracted from the testicular weight to obtain the testicular parenchyma weight. Tissue fragments were routinely dehydrated in ethanol and embedded in glycolmethacrylate (Historesin®, Leica). Semi-serial sections (3µm) were made and stained with toluidine blue/sodium borate, 1%. The

software ImageJ® was used for morphometrical analysis.

Biometry, morphometry and stereology

The gonadosomatic index (%) was calculated using the following formula *TW/BWx100* (*TW* = total testes weight, *BW* = body weight) (Amann 1970). The parenchymasomatic index was calculated as *ParW/BWx100* (*ParW* = parenchyma weight, *BW* = body weight).

The volumetric proportions of the tubular and interstitial compartments were obtained by counting 2,660 points placed on 10 digital images per animal as described by Dias et al (2019). The mass of the testicle was considered equal to its volume (Johnson et al. 1981).

The tubulesomatic index (TSI) was calculated using the formula *STV/BW x 100* (*STV* = seminiferous tubule volume, *BW* = body weight) and the epitheliumsomatic index calculated using the formula *EpV/BW x 100* (*EpV* = epithelium volume, *BW* = body weight) (Dias et al. 2019).

The tubular diameter was calculated as the average of 20 cross-sections of seminiferous tubule for each animal. The height of the seminiferous epithelium was calculated using the same tubules cross-sections, from the tunica propria to the tubular lumen. The lumen diameter was calculated after subtracting the tubular diameter from the seminiferous epithelium height.

The total length of the seminiferous tubules (STL) per testis, was estimated from previous knowledge of the volume occupied by them within the parenchyma, as well as from the average tubular diameter: $STV/\pi r^2$ (*STV* = seminiferous tubule volume; πr^2 = area of the tubule cross-section; *r* = diameter/2) (Attal 1963). The length of seminiferous tubules (per gram of testis) was calculated by dividing STL by the testes weight.

The tubular (STAr), luminal (LAr) and epithelial (EpAr) areas were calculated using the formulas: $STAr = \pi TR^2$ (TR = tubular radius), $LAr = \pi LR^2$ (LR = luminal radius), *EAr = STAr - LAr*. The tubular epithelium ratio (TER) was calculated by dividing *STAr/EAr* (Dias et al. 2019).

Germ and Sertoli cells count

In order to evaluate the populations of germ cell types located in the seminiferous epithelium in stage I of the seminiferous epithelium cycle (Amann & Schanbacher 1983, Swierstra 1968), twenty tubule cross-sections were used for each animal to count the following cell types: type A spermatogonia (SGA); spermatocytes at pre-leptotene/leptotene (PL/L) and pachytene (P), round spermatids (RS), and Sertoli cells (SC). The results were corrected for the nuclear/nucleolar diameter and the thickness of the histological section (Amann & Almquist 1962). The diameters of 30 nuclei/nucleoli of the mentioned cell types were measured for each animal. Furthermore, to evaluate the efficiency of the spermatogenic process and the support capacity of the Sertoli cells, the following ratios were calculated: efficiency of the mitotic process ((PL/L)/SGA); spermatogenesis yield (RS/SGA), meiotic index (RS/P), Sertoli cell index (RS/S), total support capacity of the Sertoli cell (SGA+PL/L+P+RS/S).

The number of Sertoli cells was estimated from the corrected number of Sertoli nucleoli and the total length of seminiferous tubules per testis (Courot et al. 1970). From this calculation, the number of Sertoli cells per gram of testis was estimated.

The daily sperm production (DSP) was estimated using the seminiferous tubule volume, the number of round spermatids, the seminiferous tubule cross-sectional area in stage 1, the duration of the cycle of the seminiferous epithelium, and the thickness of the histological section according to Amann (1970).

Leydig cell morphometry and stereology

The nuclear diameter of the Leydig cell was measured in 30 cells per animal (400X magnification). Cells with spherical nuclei were considered, from which the nuclear volume was calculated ($VN = 4/3\ \pi R^3$, R = nuclear radius). Leydig's cytoplasmic volume was calculated using the formula *VC =% cytoplasm x NuV /% nucleus*. The volume of each Leydig cell was calculated from the sum of the NuV and the VC.

The Leydig cell volume per testis calculated using the formula LC_{vol} = *Leydig cells (%) in the parenchyma x parenchyma weight / 100*. The number of Leydig cells per testis was calculated using the formula $LC_n = LC_{vol}$ / *volume of each Leydig cell*. The total number of Leydig cells per gram of testis was calculated using the formula $LC_{n/g} = LC_{vol/g}$ / *volume of each Leydig cell* ($LC_{n/g}$= number of Leydig cells per gram of testis). The Leydigsomatic index (LSI, %) was calculated by the formula $LSI = LC_{vol}$ / *BW x 100* (*BW* = body weight).

Oxidative stress

Briefly, the testicular tissue was homogenized in potassium phosphate buffer (pH 7.4, 0.2 M) with EDTA 1M, and centrifuged (13,800g, 4°C, 10 min). The supernatant was used for the calculation of superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST) levels. Data were normalized concerning the total protein levels in the supernatant.

The enzymatic activity was determined by duplicate, using a spectrophotometer (UV-Mini 1240, Shimadzu) or ELISA reader (Thermo Scientific, Waltham, MA, USA). CAT activity was assessed by measuring the decomposition rate of H_2O_2 (Aebi 1984). SOD activity was determined according to Siddiqui et al. (2005). GST activity was analyzed through the formation of 1-chloro-2,4-dinitrobenzene conjugate (CDNB) (Habig et al. 1974). The total protein concentration was measured using bovine serum albumin as a standard curve (Lowry et al. 1951) and used to homogenize stress data.

Sperm parameters

The caput/corpus and cauda regions were sliced and homogenized as previously described for the testis. Homogenization resistant spermatids (stage 19 of spermatogenesis) in testis and spermatozoa in the caput/corpus and cauda regions of the epididymis were counted according to Dias et al. (2019). To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 4.84 (the number of days that spermatids are present in the seminiferous tubules of mice). Epididymal transit time was determined by dividing the number of sperm in each region by the daily sperm production (DSP) (Dias 2018).

Statistics

The mean results were tested for normality using the Shapiro Wilk test. Parametric data were analyzed by ANOVA and the Student-Newman-Keuls post hoc test. Pearson's correlation was used to assess the relationship between two variables. Differences were significant when $P < 0.05$.

The principal component analysis (PCA) was performed to verify clustering, to eliminate redundancies, and to define the most important variables during the separation of experimental groups. To do so, data were transformed (ranging) for standardization due to different scale magnitudes. The level of importance of each variable was determined by eigenvector values (McGarigal et al. 2000), with substantial correlation values determined for each attribute concerning the principal components (PC) 1 and 2. The level of importance of each PC was determined by the Broken-stick method, where eigenvalues exceeding the expected values were

kept for interpretation. Analyzes were performed using the Fitopac 2.1.2.85 software (Shepherd 2010). In addition, the relative importance of the evaluated characteristics was calculated (Singh 1981). All analyzes were performed using the Genes software (Cruz 2008).

RESULTS

Biometry, morphometry and stereology

Foxn1$^{+/-}$ and *Foxn1*$^{-/-}$ animals showed lower body, testicular, and parenchyma weight (*P<0.05).* In addition, the PSI and GSI were significantly lower in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals (Figure 1). *Foxn* $1^{+/+}$ animals show normal tissue microstructure, with well-structured epithelia and normal interstitium. In *Foxn* $1^{+/-}$ animals, it is possible to see reduced vacuoles in the seminiferous epithelium and Leydig cell nucleus. The same can be seen in *Foxn1*$^{-/-}$ animals, although with greater alterations in the epithelial structure, larger vacuoles, and in higher proportions, causing epithelial disorganization in some seminiferous tubules (Figure 1).

The weight of the gonad showed a strong positive correlation with the GSI (r = 0.96) and the weight of the parenchyma (r = 0.92) in addition to a strong negative correlation with

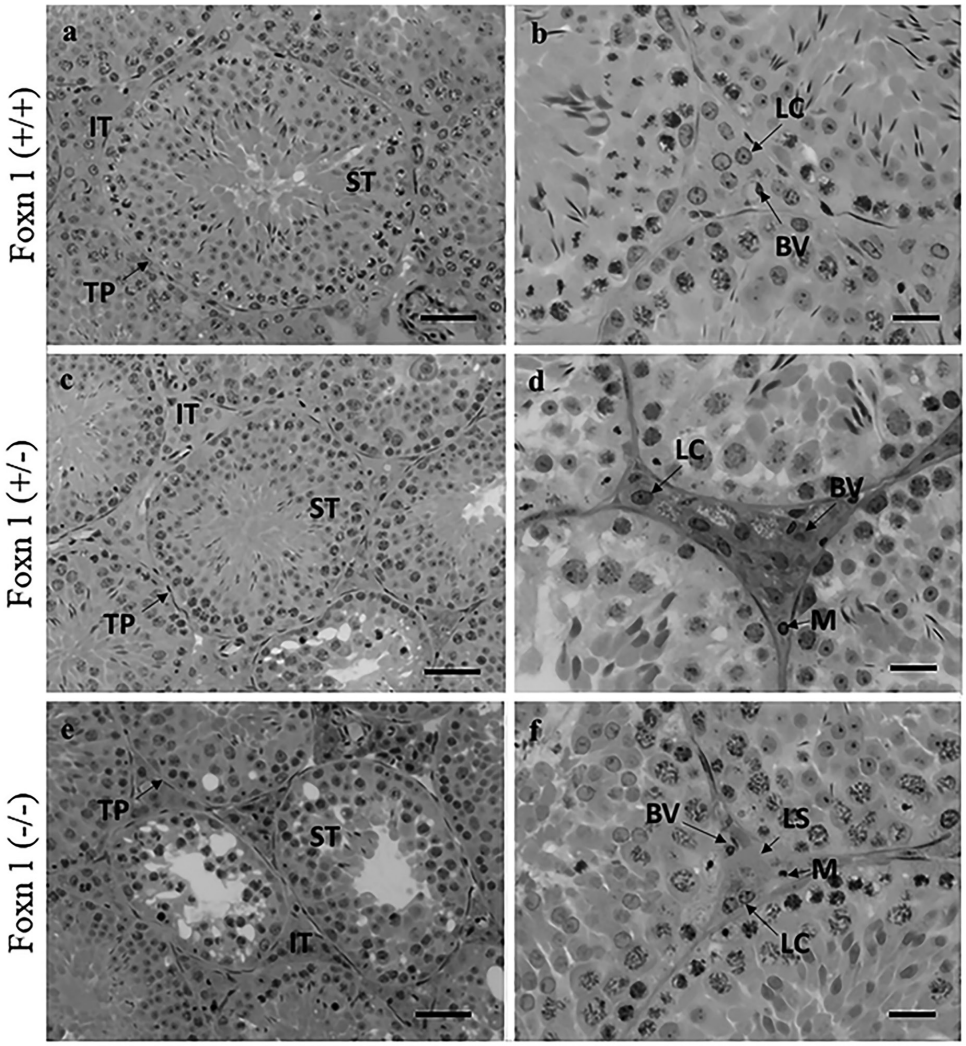

Figure 1. Histology of the testis. a, c, e = Seminiferous tubules cross-sections. b, d, f = interstitium and its components. ST: seminiferous tubule, IT: interstitium, TP: tunica propria, LC: Leydig cell, BV: blood vessel, M: macrophage, LS: lymphatic space. Bars: 50 µm (a, c, e); 20 µm (b, d, f).

the weight of the albuginea (r = -0.91) and PSI (r = -0.90). The proportion of seminiferous tubules and interstitium was not different between groups. However, the proportion of seminiferous epithelium was lower in *Foxn1*$^{-/-}$ animals, while the proportion of tunica propria was also lower in *Foxn1*$^{+/-}$ animals (Figure 2). The proportion of epithelium showed a strong positive correlation with the seminiferous tubule's proportion (r = 0.85). Tubulesomatic and epitheliumsomatic indexes were lower in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals (Table I). Both indexes showed a positive correlation with the seminiferous tubule's proportion (r = 0.52).

The diameter of the seminiferous tubule and the height of the epithelium were lower in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals, the latter having the lowest values, which was reflected in the decrease in the area of such compartments. The diameter of seminiferous tubule and the height of seminiferous epithelium show a strong positive correlation between them (r = 0.88). On the other hand, the lumen diameter and the tubule-epithelium relationship did not differ between groups (Table II). The length of seminiferous tubule per testicle and per gram of testicle was lower in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals (Table II).

Germ and Sertoli cells count

The corrected number of spermatogonia, primary spermatocytes in pre-leptotene and pachytene, and round spermatids per tubule cross-section were lower in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$. In addition, the corrected germ cells number per tubule cross-section was lower in these same groups, with *Foxn1*$^{-/-}$ animals showing the lowest mean (Table III). Sertoli cell number was not different between groups, as was the mitotic index. However, the meiotic index, the Sertoli cell index, and the Sertoli cell support capacity were lower in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$, with the lowest means observed in *Foxn1*$^{-/-}$. The number of Sertoli cells per testis and per gram of testis, as well as the spermatogenic yield, and the sperm production per testis and per gram of testis were lower in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ compared to *Foxn1*$^{+/+}$ individuals (Table III) .

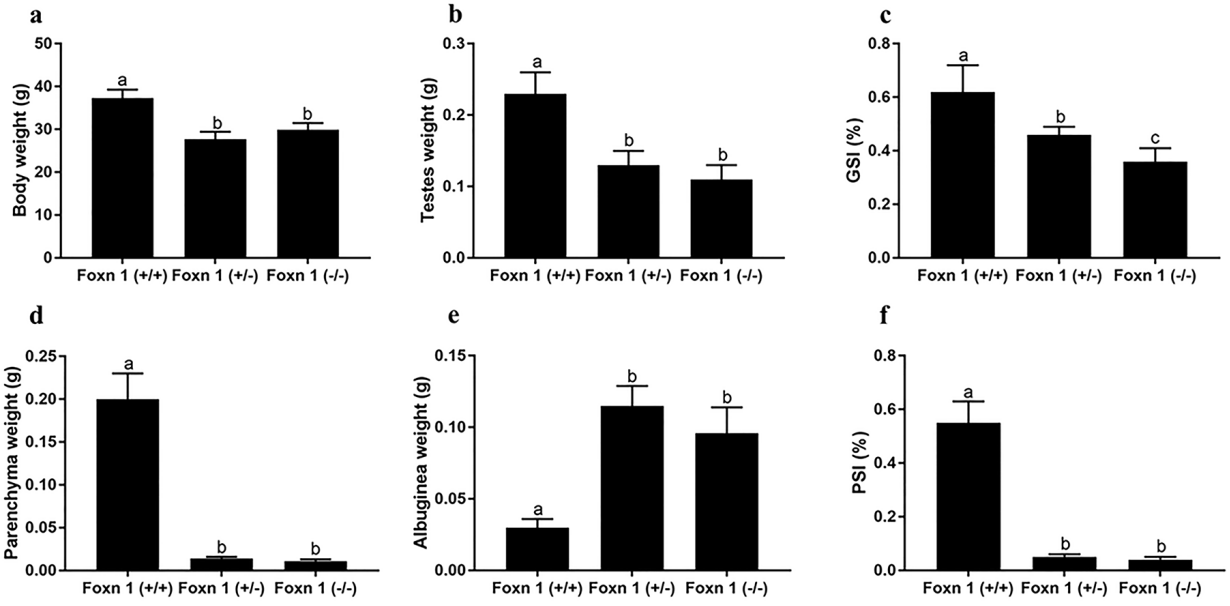

Figure 2. Body and testicular biometry. Different letters (a,b,c) are significantly different (*P<0.05*).

Table I. Proportions and indexes of the seminiferous tubules components.

Parameters	*Foxn1*$^{+/+}$	*Foxn1*$^{+/-}$	*Foxn1*$^{-/-}$
Tubule (%)	91.79±1.58 [a]	91.10±1.74 [a]	88.78±1.18 [a]
Interstitium (%)	8.21±1.02 [a]	8.90±1.74 [a]	11.22±1.8 [a]
Epithelium (%)	85.22±1.16 [a]	80.64±3.81 [a]	73.78±3.08 [b]
Tunica propria (%)	2.64±0.34 [a]	1.70±0.09 [b]	1.87±0.31 [b]
Lumen (%)	3.93±0.69 [a]	8.76±2.21 [b]	13.13±2.41 [c]
TSI (%)	0.50±0.08 [a]	0.05±0.005 [b]	0.03±0.01 [b]
ESI (%)	0.47±0.08 [a]	0.04±0.005 [b]	0.03±0.005 [b]

ESI= epitheliumsomatic index, TSI= tubulesomatic index. Different superscripts ([a,b]) in the same row are *P<0.05*. Values are mean ± S.D. ANOVA with SNK *post hoc* test.

Table II. Seminiferous tubules morphometry and stereology.

Parameters	*Foxn1*$^{+/+}$	*Foxn1*$^{+/-}$	*Foxn1*$^{-/-}$
TD (µm)	206.54±11.35 [a]	182.79±21.15 [b]	141.37±17.49 [c]
EH (µm)	84.38±2.32 [a]	66.22±2.87 [b]	55.74±4.60 [c]
LD (µm)	37.79±6.87 [a]	23.01±17.97 [a]	29.88±13.85 [a]
STL/t (m)	5.58±0.56 [a]	0.50±0.11 [b]	0.62±0.17 [b]
STL/gt (m)	24.22±2.20 [a]	4.00±1.37 [b]	5.97±1.84 [b]
TAr (µm^2)	33569.15±3740.24 [a]	26510.24±5890.93 [b]	15880.90±1723.50 [c]
LAr (µm^2)	1150.55±439.89 [a]	2192.99±1352.61 [a]	821.39±745.62 [a]
EAr (µm^2)	32418.60±3306.92 [a]	24317.26±4633.38 [b]	15059.51±3242.39 [c]
TER	1.22±0.03 [a]	1.38±0.13 [a]	1.27±0.12 [a]

TD= tubule diameter, EH= epithelium height, LD= lumen diameter, STL/t= seminiferous tubule length per testicle, TAr= tubule area, LAr= lumen area, EAr= epithelium area, TER= tubular epithelium ratio. Different superscripts ([a,b,c]) in the same row are *P<0.05*. Values are mean ± S.D. ANOVA with SNK *post hoc* test.

Sperm transit

The number of spermatids per testis was lower in the *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ groups, however, the relative number per testis was lower only in *Foxn1*$^{-/-}$ (*P<0.05*, Table IV). The daily sperm production and the relative sperm count in all epididymal segments were lower in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals (*P<0.05*), although alterations in the transit time were only noticed in the cauda epididymis of the *Foxn1*$^{-/-}$ group (Table IV). The number of sperm in the cauda epididymis per organ (r = 0.93) or per gram of organ (r = 0.92) showed a strong positive correlation with the transit time in each segment. The number of spermatids per testis (r = 1.00) and per gram of testis (r = 0.91) show a positive correlation with sperm production.

Leydig cells morphometry and stereology

The nucleus and cytoplasm volume (µm^3) were diminished in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals. The LSI followed the same behavior (*P<0.05,* Table

Table III. Germ and Sertoli cells count in seminiferous tubules cross-sections (Stage I of the seminiferous epithelium cycle).

Parameters	*Foxn1*$^{+/+}$	*Foxn1*$^{+/-}$	*Foxn1*$^{-/-}$
Spermatogonia	1.54±0.46^{a}	0.96±0.13^{b}	0.77±0.26^{b}
PL/L	26.63±1.66^{a}	16.17±2.05^{b}	14.28±3.53^{b}
P	26.22±2.74^{a}	15.79±0.99^{b}	13.68±1.56^{b}
Round spermatid	66.90±11.69^{a}	35.20±1.35^{b}	23.88±1.36^{c}
Germ Cells	121.28±15.82^{a}	68.12±4.17^{b}	52.60±0.81^{c}
Sertoli cell	4.89±0.62^{a}	5.01±0.55^{a}	6.51±0.26^{a}
Mitotic index	19.31±8.57^{a}	17.22±3.05^{a}	20.37±8.79^{a}
Meiotic index	2.54±0.20^{a}	2.23±0.10^{b}	1.75±0.11^{c}
SC index	13.89±2.95^{a}	7.51±1.26^{b}	3.67±0.12^{c}
SC support capacity	25.20±4.68^{a}	14.37±2.31^{b}	8.10± 0.32^{c}
SC/testis (x10^{6})	9.06±1.32^{a}	8.30±2.62^{b}	1.35±0.46^{b}
SC/g testis (x10^{6})	39.82±8.06^{a}	6.55±2.94^{b}	12.90±4.36^{b}
Spermatogenic yield	125.49±33.24^{a}	5.85±1.29^{b}	5.00±1.86^{b}
DSP/testis (x10^{6})	13.94±3.69^{a}	0.65±0.14^{b}	0.55±0.20^{b}
DSP/g testis (x10^{6})	59.92±11.99^{a}	5.16±1.84^{b}	5.28±1.93^{b}

PL/L = primary spermatocyte in pre leptotene/leptotene. P = primary spermatocyte in pachytene. SC= Sertoli cell. DSP= daily sperm production Values are mean ± SD. Different superscripts (^{a,b,c}) in the same row are *P<0.05*. Values are mean ± S.D. ANOVA with SNK *post hoc* test.

V). The data referring to the Leydig cell showed a slight negative correlation with the percentage of interstitium, such as Leydig volume (r = -0.59), Leydig / testicle volume (r = -0.55), Leydig nucleus volume (r = -0.53) and Leydig cytoplasm volume (r = -0.53) (Table V).

Oxidative stress

Glutathione levels were not different between groups; however, SOD and CAT levels were significantly lower in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals, with a strong correlation between them (r = 0.96, Figure 3)

Principal component analysis

The total data variation was of 82.94%, with the most important attributes of the group with correlation values > 0.6 (Figure 4a. For PC1 (horizontal axis), the most relevant attributes and their correlation values were Body weight (0.1612), Testes weight (0.1741), Albuginea weight (-0.1605), Parenchyma weight (0.1799), GSI (0.1591), Epithelium height (0.1711), Tubule diameter (0.1359), Epithelium proportion (0.1386), Tunica propria proportion (0.1541), Lumen proportion (-0.1517), Tubule volumes (0.1798), Epithelium volume (0.1798), Tunica propria volume (0.1754), Lumen volume (0.1777), TSI (0.1796), STL (0.1784), STL/g testis (0.1710), ESI (0.1796), PSI (0.1796) and

Table IV. Spermatids and sperm count in testis and epididymis.

Parameters	*Foxn1*$^{+/+}$	*Foxn1*$^{+/-}$	*Foxn1*$^{-/-}$
Spermatid number (x10^6/testis)	8.65±0.95 [a]	5.62±0.54 [b]	3.74±0.38 [b]
Spermatid number (x10^6/g testis)	175.63±21.89 [a]	121.31±14.5 [a]	64.68±11.07 [b]
DSP (x10^6/testis)	1.79±0.20 [a]	1.16±0.11 [b]	0.77±0.008 [b]
DSP (x10^6/g testis)	28.29±2.29 [a]	16.69±0.93 [b]	11.76±2.14 [b]
Sperm number (x10^6)			
Caput/corpus epididymis	3.20±1.34 [a]	2.24±0.32 [a]	0.88±0.35 [b]
Caput/corpus epididymis (per gram)	169.79±133.62 [a]	102.08±14.52 [b]	52.92±19.43 [b]
Cauda epididymis	2.71±0.36 [a]	1.52±0.09 [b]	0.42±0.06 [b]
Cauda epididymis (per gram)	185.83±30.57 [a]	139.17±17.98 [b]	28.13±3.89 [b]
Sperm transit time = caput/corpus epididymis (days)	1.85±0.84 [a]	1.92±0.16 [a]	1.12±0.54 [a]
Sperm transit time = cauda epididymis (days)	1.53±0.27 [a]	1.31±0.07 [a]	0.52±0.08 [b]

DSP: daily sperm production. *n*= 4 males/ group. Different superscripts ([a,b]) in the same row are *P<0.05*. Values are mean ± S.D. ANOVA with SNK *post hoc* test.

Seminiferous tubule area (0.1400), seminiferous epithelium area (0.1508), interstitium proportion (-0.1242), interstitium volume (0.1770), Leydig nucleus diameter (0.1678), Leydig nucleus volume (0.1705), Leydig cytoplasm volume (0.1760), Leydig cell volume (0.1757), Leydig volume/testis (0.1753), Leydig cell volume/g testis (0.1707), LSI (0.1727), Spermatid number / testis (0.1917), Spermatid number /g testis (0.1868), DSP / testis (0.1917), DSP /g testis (0.1941), Sperm number caput/ corpus epididymis (0.1560), Sperm number caput/corpus epididymis/ gram (0.1880), Sperm number cauda epididymis (0.1876), Sperm number cauda epididymis/ gram (0.1715), sperm transit time = cauda epididymis (0.1582), SOD (0.1762) and CAT (0.1796). The separation of *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ from *Foxn1*$^{+/+}$ was evidenced by changes in tubular and intertubular parameters, in addition to CAT and SOD activity (Figure 4a).

In PC2 (vertical axis), the parameters responsible for separating the groups were tubule diameter (-0.2721); lumen diameter (-0.40553277), tubule proportion (-0.25002745), tubule area (-0.2665), and lumen area (-0.411403319) and TER (-0.3521) (Figure 4a).

The relative importance of the variables

The relative importance of the variables pointed out that all variables were equally important for the separation of the experimental groups (Figure 4b). Removing one of the variables would alter the group's distribution.

DISCUSSION

Although *Foxn1*$^{-/-}$ mice are widely used in reproduction studies, there are few reports with details regarding its reproductive biology. Thus, in the present study, we evaluated the testicular parenchyma of wild-type, *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ mice.

The reduction in body weight observed in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals is common when dealing with different species, however,

Table V. Leydig cell morphometry and stereology.

Parameters	*Foxn1*$^{+/+}$	*Foxn1*$^{+/-}$	*Foxn1*$^{-/-}$
Leydig nucleus (%)	0.80±0.32 [a]	1.10±0.18 [a]	1.49±0.25 [a]
Leydig cytoplasm (%)	4.73±0.86 [a]	6.14±1.35 [a]	7.70±0.73 [a]
Leydig cell (%)	5.53±1.17 [a]	7.24±1.50 [a]	9.20±0.95 [a]
Nucleus diameter (µm)	6.55±0.30 [a]	4.54±0.56 [b]	4.49±0.29 [b]
Nucleus volume (µm^3)	147.64±20.30 [a]	50.88±18.97 [b]	47.74±9.56 [b]
Cytoplasm volume (µm^3)	926.56±212.99 [a]	284.22±109.45 [b]	247.31±39.19 [b]
LC volume (µm^3)	1074.20±226.03 [a]	335.11±127.62 [b]	295.05±47.33 [b]
LC / testis (x10^6)	11.41±3.05 [a]	2.47±0.74 [b]	3.03±1.46 [b]
LC / g testis (x10^6)	51.06±21.88 [a]	19.34±5.60 [b]	30.57±17.14 [a]
LSI (%)	0.029±0.005 [a]	0.004±0.001 [b]	0.003±0.001 [b]

LC = Leydig cell, LSI: leydigsomatic index. Different superscripts ([a,b]) in the same row are *P<0.05*. Values are mean ± S.D. ANOVA with SNK *post hoc* test.

significant reductions in testicular weight and consequent parenchyma weights, and in the parenchymasomatic index, may indicate changes in spermatogenesis, thus in sperm production (França & Russell 1998). GSI and PSI were reduced in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ groups, indicating that important changes within the organ are taking place.

The proportion between the spermatogenic and steroidogenic compartments is quite variable in mammals (França & Russell 1998), being one of the main factors responsible for variation in sperm production among species. The tubular compartment constitutes the major part of the testicular parenchyma, ranging from 70 to 90% of the whole organ area and all values observed in the present study remained within this range (Clermont & Trott 1969). The seminiferous tubules show a direct relationship with the tubular length, as well as with germ and Sertoli cells' populations, culminating in sperm production (França & Russell 1998).

The seminiferous epithelium proportion was lower in *Foxn1*$^{-/-}$ animals than in the *Foxn1*$^{+/+}$ *or Foxn1*$^{+/-}$, consequently reducing the epitheliumsomatic index in *Foxn1*$^{-/-}$ animals. Although in *Foxn1*$^{+/-}$ animals there was no significant reduction in the proportion of seminiferous epithelium, this proportion was lower, reflecting the reduction in the epitheliumsomatic index in these animals concerning *Foxn1*$^{+/+}$. Such alterations would inevitably compromise sperm production since the seminiferous epithelium shows a direct correlation with the number of germ cells and sperm produced. In addition, significant reductions in the proportions of the tunica propria both in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals were noticed. Myoid cells, which are important components of the tunica propria have a functional correlation with the Leydig cells, acting in the fine regulation of spermatogenesis (Zhou et al. 2019), thus such reduction of the *tunica propria* might influence the spermatogenic process.

The average tubular diameter does not change significantly after the establishment of sexual maturity and remains constant

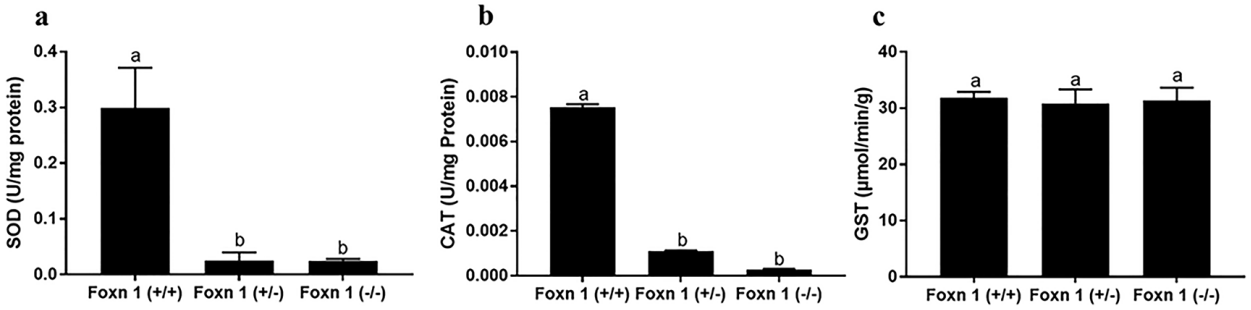

Figure 3. Oxidative stress analysis. a. Superoxide dismutase (SOD), b. Catalase (CAT), and c. Glutathione Transferase (GST) levels. Values as mean ± SD. Different letters (a,b,c) are statistically different (*P<0.05*).

throughout the seminiferous epithelium cycle, ranging from 180 to 300 µm, even though expressive interspecific variations occur (França & Russell 1998). In mice, individuals of lower body weight also show lower tubular diameter (O'Keane et al. 1986), which was observed in *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals. Interestingly, *Foxn1*$^{-/-}$ animals showed the lowest tubule diameter, also noticed for other seminiferous epithelium parameters, such as the length of the tubules, as well as in their respective areas. Due to such reductions, the spermatid's populations were significantly lower than in the other groups, reflecting in lower sperm production and the sperm number present in all epididymis' segments.

The reduction in the spermatogonia, spermatocytes and spermatids counts led to a significant reduction in the number of germ cells in both *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ animals. Such reduction is in accordance with the reduction in height of the seminiferous epithelium observed in individuals of the *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ strains. Furthermore, although the Sertoli cell count was not altered, there were lower Sertoli cell support capacity and lower Sertoli cell indexes, which culminated in a reduced sperm reserve, daily sperm production and spermatogenesis yield between *Foxn1*$^{+/-}$ and *Foxn1*$^{-/-}$ strains, as well as between both and *Foxn1*$^{+/+}$.

Despite the lower sperm count within the epididymis, the spermatic transit, which is the transport of sperm through the epididymal duct, was only altered in the cauda in *Foxn1*$^{-/-}$ animals. Such transport is dependent on the differential hydrostatic pressure gradient between the proximal and distal portions of the epididymal duct, the contractile activity of the duct wall, controlled by the autonomic nervous system, and the action of androgens (Robaire & Viger 1995, Cosentino & Cockett 1986, Klinefelter 2002). The delay in transit time through the epididymis does not alter the fertile capacity of gametes, but when it is accelerated, fertility is compromised since the time available for the processes required for the acquisition of fertile capacity is reduced (Kempinas et al. 1998). Changes in sperm transit time also change the amount of sperm available for ejaculation (Klinefelter 2002).

The individual volume and number of Leydig cells, as well as the leydigsomatic index, were reduced in *Foxn1*$^{-/-}$ and *Foxn1*$^{+/-}$ animals, which might be associated with low testosterone production. The transcription factor Foxn1 is translocated to the cell nucleus after phosphorylation, where it regulates the expression of several genes (Mecklenburg et al. 2005). Foxn1 can play a role in maintaining the physiology of the testes, however, it is poorly expressed in the nucleus of Leydig cells in *Foxn1*$^{-/-}$ animals (Oliveira et al. 2020). Thus, the cytoplasmic distribution of such transcription factor suggests its participation in the regulation

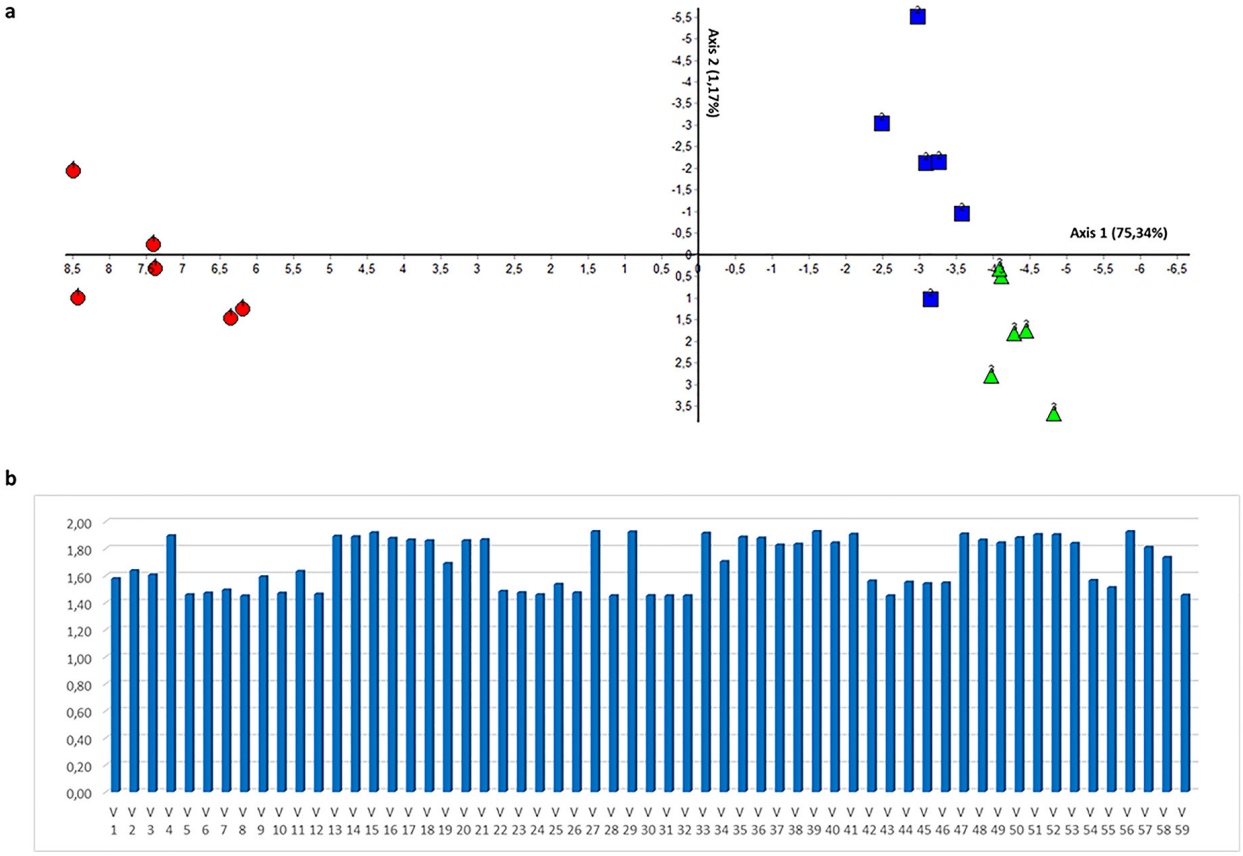

Figure 4. a. Principal component analysis. b. The relative contribution of the most important quantitative/ qualitative characteristics to the divergence between groups. Red circles: *Foxn1*$^{+/+}$, blue squares: *Foxn1*$^{+/-}$, green triangles: *Foxn1*$^{-/-}$.

of the function of the steroidogenic pathway in Leydig cells (Oliveira et al. 2020).

Both *Foxn1*$^{-/-}$ and *Foxn1*$^{+/-}$ animals showed reduced SOD and CAT activity. SOD is an antioxidant enzyme considered to be the first line of defense against the deleterious effects of reactive oxygen species (ROS), catalyzing the reaction of conversion of the superoxide radical to H_2O_2 (Barbosa et al. 2010) which is easily degraded by CAT and GST (Aitken & Roman 2008). CAT converts H_2O_2 to water and oxygen (Barreiros et al. 2006). Glutathione levels did not differ between groups. GST is involved in the reactions of phase II of antioxidant activity, reducing the production of lipid peroxidation through the reduction of hydroperoxides (Hayes et al. 2005). Thus, it is responsible for cellular detoxification through glutathione conjugated with xenobiotics and aldehyde products produced in lipid peroxidation, making them more soluble in water (Habig et al. 1974). Its activity, as well as that of CAT, can be directly inhibited by high concentrations of NO (Kostic et al. 2000, Wong et al. 2001). Thus, we can suggest that *Foxn1*$^{-/-}$ and *Foxn1*$^{+/-}$ animals have an inherent deficiency in natural antioxidant defenses, which can cause changes to reproductive functionality (the quality and quantity of sperm), showing increased structural changes in the seminiferous epithelium.

CONCLUSIONS

Foxn1$^{+/-}$ and *Foxn1*$^{-/-}$ strains resulting from the mating between *Foxn1*$^{-/-}$ male and heterozygous Balb/c female, are quite similar

concerning reproductive parameters. They show compromised spermatogenesis, with reduced spermatids' population within the also reduced seminiferous epithelium, reflecting in low daily sperm production, especially on the *Foxn1*$^{-/-}$ animals. Such behavior might be related to the lower Leydig cell volume and populations within the interstitium, therefore affecting the fertility rate of the strains.

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REFERENCES

AEBI H. 1984. Oxygen Radicals in Biological Systems. Method Enzymol 105: 121-126.

AITKEN RJ & ROMAN SD. 2008. Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longev 1: 15-24.

ALTEN HE & GROSCURTH P. 1975. The postnatal development of the ovary in the "nude" mouse. Anat Embryol 148: 35-46.

AMANN R. 1970. The male rabbit. IV. Quantitative testicular histology and comparisons between daily sperm production as determined histologically and daily sperm output. Fertil Steril 21: 662-672.

AMANN R & ALMQUIST J. 1962. Reproductive capacity of dairy bulls. VIII. Direct and indirect measurement of testicular sperm production. Dairy Sci 45: 774-781.

AMANN RP & SCHANBACHER BD. 1983. Physiology of male reproduction. J Anim Sci 380-403.

ATTAL JCM. 1963. Développement testiculaire et établissement de la spermatogenèses chez le taureau. Ann Biol Anim Biochim Biophys 3: 219-241.

BARBOSA KBF, COSTA NMB, DE CÁSSIA GONÇALVES ALFENAS R, DE PAULA SO, MINIM VPR & BRESSAN J. 2010. Estresse oxidativo: Conceito, implicações e fatores modulatórios. Rev Nut 23: 629-643.

BARREIROS ALBS, DAVID JM & DAVID JP. 2006. Estresse oxidativo: Relação entre geração de espécies reativas e defesa do organismo. Quim Nova 29: 113-123.

BRISSETTE JL, LI J, KAMIMURA J, LEE D & DOTTO GP. 1996. The product of the mouse nude locus, whn, regulates the balance between epithelial cell growth and differentiation. Genes Develop 10: 2212-2221.

BRÜNNER N, SVENSTRUP B, SPANG-THOMSEN M, BENNETT P, NIELSEN A & NIELSEN J. 1986. Serum steroid levels in intact and endocrine ablated BALB/C nude mice and their intact littermates. J Steroid Biochemist 25: 429-432.

CLERMONT Y & TROTT M. 1969. Duration of the cycle of the seminiferous epithelium in the mouse and hamster determined by means of 3H-thymidine and radioautography. Fertil Steril 20: 805-817.

COSENTINO MJ & COCKETT ATK. 1986. Review Article: Structure and Function of the Epididymis. Urol Res 14: 229-240.

COUROT M, HOCHEREAU-DE-REVIERS M & ORTAVANT R. 1970. Spermatogenesis. In JOHNSON AD ET AL. (Eds), The testis, New York: Academic Press, New York, USA, p. 339 -432.

CRUZ CD. 2008. Programa Genes - Diversidade Genética. Viçosa: Editora UFV, 71 p.

DANEVA T, SPINEDI E, HADID R & GAILLARD RC. 1995. Impaired hypothalamo-pituitary-adrenal axis function in swiss nude athymic mice. Neuroendocrinol 62: 79-86.

DIAS FCR. 2018. Extrato hidroalcoólico da raiz de *Pfaffia glomerata* (spreng.) Pedersen interfere em parâmetros reprodutivos e fertilidade de camundongos machos adultos. Universidade Federal de Viçosa, 157 p.

DIAS FCR, MARTINS ALP, DE MELO FCSA, CUPERTINO MDOC, GOMES MDELM, DE OLIVEIRA JM & DA MATTA SLP. 2019. Hydroalcoholic extract of *Pfaffia glomerata* alters the organization of the seminiferous tubules by modulating the oxidative state and the microstructural reorganization of the mice testes. J Ethnopharmacol 233: 179-189.

FLANAGAN SP. 1966. 'Nude', a new hairless gene with pleiotropic effects in the mouse. Genet Res 8: 295-309.

FRANÇA LR & RUSSELL LD. 1998. The testis of domestic mammals. In MARTÍNEZ-GARCIA F & REGARDERA J (Eds), Male reproduction - a multidisciplinary overview, Madrid, Spain, p. 198-219.

GOYA RG, REGGIANO PC, VESENBECKH SM, PLÉAU JM, SOSA YE, CÓNSOLE GM & DARDENNE M. 2007. Thymulin gene therapy prevents the reduction in circulating gonadotropins induced by thymulin deficiency in mice. Am J Physiol Endocrinol Metab 29: E182 E187.

HABIG WH, PABST MJ & JAKOBY WB. 1974. Glutathione S-Transferases the first enzymatic step in mercapturic acid formation. J Biol Chemist 249: 7130-7140.

HAYES JD, FLANAGAN JU & JOWSEY IR. 2005. Glutathione Transferases. Annu Rev Pharmacol Toxicol 51-88.

JOHNSON L, PETTY CS & NEAVES WB. 1981. A New Approach to Quantification of Spermatogenesis and Its Application to Germinal Cell Attrition During Human Spermiogenesis1. Biol Reprod 25: 217-226.

KARNOVSKY MJ. 1965. A formaldehyde-glutaraldehyde fixative of righ osmolality for use in electron microscopy. J Cell Biol 27: 137.

KEMPINAS W, SUAREZ J, ROBERTS N, STRADER L, FERRELL J & GOLDMAN J. 1998. Rat epididymal sperm quantity, quality and transit time after guanethidine-induced sympathectomy. Biol Reprod 59: 890-896.

KLINEFELTER GR. 2002. Actions of toxicants on the structure and function of the epididymis. In ROBARIE B & HINTON BT (Eds), The Epididymis – from molecules to clinical practice, New York, USA, p. 353-369.

KOSTIC TS, ANDRIC SA, MARIC D, & KOVACEVIC RZ. 2000. Inhibitory effects of stress-activated nitric oxide on antioxidant enzymes and testicular steroidogenesis. J Steroid Biochemist Mol Biol 75: 299-306.

LOWRY OH, ROSEBROUGH NJ, FARR L & RABDALL RJ. 1951. protein measurement with the folin phenol reagent. Anal Biochem 193: 265-275.

MCGARIGAL K, CUSHMAN SA & STAFFORD SG. 2000. Multivariate statistic for wildlife and ecology research. Springer-Verlag, 76 p.

MECKLENBURG L, TYCHSEN B & PAUS R. 2005. Learning from nudity: Lessons from the nude phenotype. Exp Dermatol 14: 797-810.

NAGASAWA H & YANAI R. 1977. Mammary growth and function and pituitary prolactin secretion in female nude mice. Act Endocrinol 86: 794-802.

NEHLS M, KYEWSKI B, MESSERLE M, WALDSCHÜTZ R, SCHÜDDEKOPF K, SMITH AJ & BOEHM T. 1996. Two genetically separable steps in the differentiation of thymic epithelium. Science 272: 886-889.

O'KEANE JC, BRIEN TG, HOOPER ACB & GRAHAM A. 1986. Testicular Activity in Mice Selected for Increased Body Weight. Andrologia 18: 190-195.

OLIVEIRA CF, LARA NL, LACERDA SM, RESENDE RR, FRANÇA LR & AVELAR GF. 2020. Foxn1 and Prkdc genes are important for testis function: evidence from nude and scid adult mice. Cell Tis Res 380: 615-625.

OLSEN NJ, OLSON G, VISELLI SM, GU X & KOVACS WJ. 2001. Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. Endocrinol 142: 1278- 1283.

PIERPAOLI W & BESEDOVSKY HO. 1975. Thymus involvement in female sexual maturation. Clin Exp Immunol 20: 323-338.

REBAR RW, MORANDINI IC, PETZE JE & ERICKSON GF. 1982. Hormonal Gonadotropins. Biol Reprod 27: 1267-1276.

ROBAIRE B & VIGER RS. 1995. Regulation of epididymal epithelial cell functions. Biol Reprod 52: 226-236.

RYGAARD J & FRIIS CW. 1974. The husbandry of mice with congenital absence of the thymus (nude mice). Z Versuchstierkd 16: 1-10.

SHEPHERD GJ. 2010. Fitopac 2.1.2.85. Manual Do Usuário. Campinas, v. 1, 5 p.

SIDDIQUI IA, RAISUDDIN S & SHUKLA Y. 2005. Protective effects of black tea extract on testosterone induced oxidative damage in prostate. Cancer Lett 227: 125-132.

SINGH D. 1981. The relative importance of characters affecting genetic divergence. Indian J Genet Plant Breed 41: 237-245.

SWIERSTRA EE. 1968. Cytology and duration of the cycle of the seminiferous epithelium of the boar; duration of spermatozoan transit through the epididymis. The Anatomical Record 161: 171-185.

WONG PS, EISERICH JP, REDDY S, LOPEZ CL, CROSS CE & VAN DER VLIET A. 2001. Inactivation of glutathione S-transferases by nitric oxide-derived oxidants: exploring a role for tyrosine nitration. Arch Biochem Biophys 394: 216-228.

ZHOU R, WU J, LIU B, JIANG Y, CHEN W, LI J, QUANYUAN H & HE Z. 2019. The roles and mechanisms of Leydig cells and myoid cells in regulating spermatogenesis. Cell Mol Life Sci 76: 2681-2695.

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FCRD, MBS, ELO, FCSAM, NAP: experiments, data analysis and data discussion, writing. SLPM, AOG, MLMG: project design, data discussion, manuscript writing and reviewing.