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Original Article

Analysis of male reproductive parameters in a murine model of mucopolysaccharidosis type I (MPS I)

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Abstract: Mucopolysaccharidosis (MPS) I is a lysosomal storage disorder (LSD) that is characterised by alpha-L-iduronidase (Idua) deficiency and continuous deposition of glycosaminoglycans (GAGs), which consequently interferes with cell signalling mechanisms and results in multisystemic and progressive symptoms. The animal model of MPS I (*Idua-/-*) has been widely studied to elucidate the consequences and progression of the disorder; however, studies specifically assessing the male reproductive tract are lacking. The aim of this study was to evaluate some of the reproductive characteristics of male MPS I mice in two phases of life. Reproductive organ biometry, sperm counts, sperm morphological evaluation, plasma testosterone measurements and histopathological, histomorphometrical and immunohistochemical analysis were performed in 3- and 6-month-old C57BL/6 *Idua+/+* and *Idua-/-* mice. Seminal vesicle weights were decreased in both the 3- and 6-month-old *Idua-/-* mice. Decrease in sperm counts and the majority of the histopathological signs were observed in the 6-month-old *Idua-/-* mice. No differences were detected in the sperm morphological analysis. Immunohistochemistry revealed that seminiferous tubules from 3-month-old *Idua-/-* mice were more intensely stained with anti-caspase-3 than 3-month-old *Idua+/+* mice, but no difference was found at 6 months. These results suggest that MPS I interferes with male reproductive parameters both in 3 and 6-month-old animals and histopathological signs are more pronounced in 6-month-old mice, indicating that the effects of the disorder may intensify with the disease progression.

Keywords: Knockout mice, mucopolysaccharidosis, spermatogenesis, testis

Introduction

Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disorder (LSD) characterised by the continuous deposition of glycosaminoglycans (GAGs) as a consequence of deficiency in α-L-iduronidase (Idua), a lysosomal enzyme that hydrolyses heparan and dermatan sulphate. The improper storage of GAGs interferes with signalling processes and results in multisystemic and progressive symptoms [1].

Due to high phenotypic variability, MPS I can be classified into the following 3 groups according to its severity: Hurler, Hurler-Scheie and Scheie syndromes. The main symptoms of Hurler syndrome are growth deficiency, joint stiffness, coarse facial appearance, mental retardation, speech impairment, hepatosplenomegaly, and respiratory and cardiovascular problems, resulting in a life expectancy of 10 years [1]. Murine models of MPS I (*Idua-/-*) manifest similar symptoms and are considered to simulate Hurler syndrome in which α-L-iduronidase is completely inactive [2].

A set of studies have reported associations between reproductive damage and exogenous factors, such as pollutants and drugs [3, 4], and endogenous factors, such as congenital abnormalities [5, 6], demonstrating that the reproductive tract is sensitive to the liability of adverse conditions.

Some studies have demonstrated low reproductive efficiency in patients and animal models of other types of LSD, such as Gaucher [7] and Niemann-Pick diseases [8-10]. However, to date, no studies have specifically addressed reproductive parameters in male individuals affected by MPS. The aim of this study was to evaluate some of the male reproductive param-

eters of *Idua-/-* mice to contribute to the description of the animal model of MPS I.

Methods

Animals

C57BL/6 mice were kindly donated by Dr. Elizabeth Neufeld (UCLA, USA) and Dr. Nance B. Nardi (UFRGS, Brazil) and were bred from heterozygous (*Idua+/-*) matings to establish the colony at Universidade Federal de São Paulo (UNIFESP). We used the MPS I model described by Ohmi et al. [11], which is similar to that described by Clarke [2]. The *Idua-/-* genotype mimics Hurler syndrome, as the hydrolase α-L-iduronidase is completely inactive. Animals were maintained on a 12 h light/dark cycle with food and water available *ad libitum* and without any contact with females after weaning. One-month-old mice were genotyped by polymerase chain reaction using the following primers: GAGACTTGGAATGAACCAGAC (sense) and ATAGGGGTATCCTTGAACTC (antisense) [12]. The mice were distributed into four groups according to genotype (*Idua+/+* or *Idua-/-*) and age (3 or 6 months, representing young and middle-aged adults, respectively). The four groups were designated *Idua+/+*3m, *Idua-/-*3m, *Idua+/+*6m and *Idua-/-*6m.

Given the difficulty in obtaining *Idua-/-* animals, 4 to 6 animals were assigned per group. Euthanasia was performed by decapitation without any contact with other animals to promote immediate death and to minimise suffering.

Ethical approval

Experimental procedures involving animals were in accordance with the Ethical Research Committee from Universidade Federal de São Paulo (UNIFESP) in 2011 (CEP 602/11).

Biometrical analyses

Body weights were measured before euthanasia using a precision balance. Absolute weights of the testes, epididymis, ventral prostate and seminal vesicles were measured on an analytical balance after euthanasia. We have considered the mean of the absolute weights of paired organs, such as the testes and epididymis. Relative weights were calculated by dividing the absolute weights by body weights and were expressed as a percentage: [(organ weight/body weight) × 100].

Sperm counts

The estimate of daily sperm production (DSP) was adapted from Thayer *et al.* [13]. The right testis was homogenised with saline and detergent solution (NaCl 0.9% and Triton X-100 0.05%). Cells resistant to homogenisation (stages 14 to 16 of spermatogenesis) were counted in a hemocytometer chamber under a light microscope at 400 × magnification. The homogenate was diluted 1:1 (v/v). Two 5 µL aliquots were applied to the chamber, and the average of six chambers was used to estimate the number of sperm/testis. To estimate the DSP, the testicular sperm number was divided by 4.8, which corresponds to the number of days that resistant spermatids remain in the testes.

Sperm morphology

The left epididymal cauda was minced and immersed in 500 µl of phosphate buffered saline (PBS). Sperm were allowed to disperse into the buffer for 15 min at room temperature. Two 5 µl aliquots were pipetted onto a hemocytometer chamber, and 300 sperm were observed under a light microscope at 400 × magnification [14] and head and tail abnormalities were registered [15]. Afterwards, 100 sperm were counted and classified according to their integrity as normal, tail-lost sperm and head-lost sperm.

Testicular histopathology

Immediately after euthanasia, the left testis was fixed in buffered formalin (10%) for 4 h. After this period, the organ was divided into two parts, one was returned to the buffered formalin for an additional 24 h for immunohistochemical analysis and the other was placed in Alfac (85% ethanol 80%, 10% formaldehyde and 5% glacial acetic acid) for 20 h. After fixation, the piece was dehydrated with 80-100% ethanol, diaphanised with xylol and embedded in Paraplast Plus®. Cross sections of 5 µm thickness were stained with hematoxylin/eosin or toluidine blue (pH = 2.5). Seminiferous tubules cross sections were considered damaged when signs of degeneration (vacuolisation, desquamation of the seminiferous epithelium, loss of

Table 1. Biometrical parameters. Body weight and absolute and relative weight of the testes, epididymis, ventral prostate and seminal vesicles. Values are expressed as the mean and standard deviation (parentheses)

	3-month-old			6-month-old		
	Idua+/+	*Idua-/-*	p	*Idua+/+*	*Idua-/-*	p
Body weight (g)	26.8 (0.91)	25.2 (0.47)	0.056	28.1 (0.56)	27.9 (1.68)	0.895
Absolute weights						
Testis (mg)	99.1 (8.5)	110.8 (14.7)	0.161	102.0 (6.8)	110.2 (7.7)	0.115
Epididymis (mg)	33.6 (8.2)	31.6 (6.3)	0.673	37.7 (2.4)	38.2 (6.3)	0.875
Ventral prostate (mg)	11.9 (1.6)	7.6 (1.3)*	0.002	11.2 (1.5)	10.5 (4.5)	0.749
Seminal vesicle (mg)	152.2 (22.3)	99.1 (21.0)*	0.004	199.1 (27.6)	115.8 (37.8)*	0.004
Relative weights						
Testis (%)	0.35 (0.02)	0.43 (0.06)*	0.022	0.36 (0.03)	0.38 (0.03)	0.225
Epididymis (%)	0.12 (0.03)	0.12 (0.02)	0.853	0.13 (0.01)	0.13 (0.004)	0.970
Ventral prostate (%)	0.04 (0.01)	0.03 (0.005)*	0.02	0.04 (0.01)	0.03 (0.01)	0.579
Seminal vesicle (%)	0.55 (0.09)	0.38 (0.08)*	0.016	0.70 (0.12)	0.39 (0.08)*	0.001

*Idua+/+*3m: 3-month-old control group; *Idua-/-*3m: 3-month-old knockout group; *Idua+/+*6m: 6-month-old control group; *Idua-/-*6m: 6-month-old knockout group. Unpaired t-test. *$p < 0.05$ compared with same aged Idua+/+ group; n = 5 animals/group.

germ cells or presence of immature spermatids in the lumen); signs of necrosis (fragmentation or compaction of the cell nucleus) and interstitial alterations (signs of fibrosis and vacuolisation) were present. One testis cross section per animal was considered in this analysis.

To quantify the seminiferous damage, 3 non-consecutive cross sections per animal were evaluated. Damaged and normal tubular cross sections were counted [3]. To observe the possible accumulation of basophilic material, testicular sections were coloured with toluidine blue. Staining was quantitatively analysed using Image J software (National Institutes of Health, Maryland, USA). Three regions from each testicular cross section were examined, and 10 interstitial regions from each figure were evaluated to establish the colour intensity, which was expressed as the relative optical density [16].

Testicular histomorphometry

Histomorphometrical analyses were performed using Axio Vision 4.8 (Zeiss®).

Tubular diameter was measured on 30 circular tubular cross sections from each animal, regardless of the stage of the seminiferous epithelium. The epithelial height was determined from 20 tubular cross sections in stage XII, and percentage of tubular and interstitial areas was determined from 15 images (1400 µm × 1100 µm) at 200 × magnification. One testicular cross section per animal was evaluated.

Plasma testosterone levels

Blood was collected in heparinised microtubes. Plasma was separated by centrifugation (4°C/3000 rpm/10 min) and stored at -80°C. Testosterone was quantified in duplicate by chemiluminescence using automated UniCel dxI 800 (Beckman Coulter®) equipment with a sensitivity of 10 ng/dL. The intra- and inter-assay coefficients of variation were 1.99 and 4.22, respectively.

Immunohistochemistry

Immunohistochemical staining for pro-apoptotic caspase-3 and anti-apoptotic bcl2 was performed using the avidin-biotin-peroxidase complex method. Three-micron sections were deparaffinised with xylol and rehydrated with a gradient of ethanol (100%-70%). Antigen retrieval was performed by heating the sample in citrate buffer (0.01 M pH = 6.0), inhibiting endogenous peroxidase with 3% H_2O_2 (5 × 5 min) and treating sections for 30 min with 1% bovine serum albumin in PBS. Sections were incubated overnight at 4°C with rabbit polyclonal anti-cleaved caspase-3 antibody ab-52294 (Abcam®) or rabbit polyclonal anti-bcl-2 antibody ab32124 (Abcam®); both antibodies

Table 2. Characterisation of sperm, hormonal and testicular histomorphometrical parameters. Values are expressed as the mean and standard deviation (parentheses)

	3-month-old			6-month-old		
	Idua+/+	*Idua-/-*	p	*Idua+/+*	*Idua-/-*	p
Sperm counts						
Sperm number in the testis (× 10^6)	25.6 (6.7)	20.7 (3.9)	0.197	27.7 (1.5)	23.3 (2.3)*	0.007
DSP (× 10^6)	5.3 (1.4)	4.3 (0.8)	0.196	5.8 (0.3)	4.8 (0.5)*	0.007
Sperm morphology						
Abnormal sperm (%)	63.3 (6.0)	67.1 (15.7)	0.63	58.2 (5.9)	55.7 (7.8)	0.577
Tail-lost sperm (%)	9.0 (5.24)	20.87 (10.04)*	0.047	19.0 (7.17)	20.32 (7.96)	0.789
Head-lost sperm (%)	9.60 (4.51)	19.07 (8.15)	0.052	18.20 (4.76)	17.79 (4.91)	0.897
Hormonal analyses#						
Plasma testosterone (ng/dL)	1051 (299.7)	4191 (3025.0)	0.09	2402 (1413.0)	2139 (2130.0)	0.938
Testicular morphometrical parameters						
Tubular diameter (µm)	184.6 (8.6)	181.3 (10.8)	0.611	196.5 (13.8)	198.0 (12.9)	0.865
Epithelium height (µm)	56.3 (2.3)	54.1 (3.9)	0.307	54.9 (3.8)	58.7 (2.5)	0.102
Frequency of interstitial area (%)	15.19 (3.90)	32.21 (4.22)*	0.0002	20.62 (1.78)	27.03 (5.89)*	0.048

*Idua+/+*3m: 3-month-old control group; *Idua-/-*3m: 3-month-old knockout group; *Idua+/+*6m: 6-months-old control group; *Idua-/-*6m: 6-months-old knockout group. Unpaired t-test. *$p < 0.05$ compared with same aged *Idua+/+* group; n = 5 animals/group. #n = 4 in *Idua-/-* groups due to the lack of plasma volume for chemiluminescent analysis. DSP: daily sperm production.

were diluted 1:300 in 1% BSA. After the PBS washes, the sections were incubated with biotinylated secondary antibody (Dako®) followed by streptavidin (Dako®). Labelling was revealed with 3,3'-diaminobenzidine (Dako®). In negative control sections, the primary antibody was replaced by BSA. Sections were counterstained with hematoxylin.

The intensity of the immunohistochemical reaction was quantified by a blinded investigator. Ten seminiferous cross sections were evaluated per animal and classified from 0 to 3 according to a scoring scale (0 = absent; 1 = weak; 2 = moderate and 3 = intense) [17].

Statistical analysis

Statistical analysis was performed using unpaired t-test. All analyses were performed using StatSoft Statistica 7.0® and the level of significance was set at ≤ 0.05.

Results

Biometrical analyses

Body weights did not differ between *Idua+/+* and *Idua-/-* mice of the same age. The relative testicular weight was higher in *Idua-/-*3m mice compared to the controls ($P = 0.022$) (**Table 1**). The epididymal biometric parameters did not differ among the different genotypes (**Table 1**). Interestingly, the absolute and relative prostatic weights were lower in the *Idua-/-*3m group ($P = 0.002$ and 0.02, respectively). The absolute seminal vesicle weight was decreased in the *Idua-/-*3m ($P = 0.004$) and *Idua-/-*6m ($P = 0.004$) groups, as were the relative weights ($P = 0.016$ and $P = 0.001$, respectively) (**Table 1**).

Sperm analyses

The DSP, and consequently the number of sperm per testis, were significantly reduced in the *Idua-/-*6m compared with *Idua+/+*6m mice ($P = 0.007$ and $P = 0.007$, respectively). No significant difference was found among the 3-month-old mice (**Table 2**), although means are similar among 3 and 6-month-old *Idua-/-* groups. Additionally, there was no significant difference in the percentage of abnormal sperm in mice at 3 or 6 months. However, the frequency of tail-lost sperm was higher in *Idua-/-*3m group, compared to *Idua+/+*3m group (0.047) (**Table 2**).

Plasma testosterone analyses

No significant difference was detected in plasma testosterone (**Table 2**).

Testicular histopathology and histomorphometry

Signs of tubular degeneration, such as disorganisation of the seminiferous epithelium, vacuolisation and presence of tubules lined with

Table 3. Qualitative histopathologic analysis of testes

	3-month-old		6-month-old	
	Idua+/+	*Idua-/-*	*Idua+/+*	*Idua-/-*
Degeneration	1/6	1/6	2/6	4/6
Necrosis	0/6	0/6	0/6	2/6
Interstitial alteration	1/6	1/6	0/6	6/6

1 histological section/animal; n = 6 animals/group.

only Sertoli cells and characterised by few or absent germ cells, were observed in all groups (**Table 3** and **Figure 1**), especially some of the *Idua-/-*6m mice. However, the frequency of degenerated tubules was not statistically different between *Idua-/-* and *Idua+/+* mice of the same age (**Figure 1F**). Sections stained with toluidine blue suggested an accumulation of acid substrate, possibly GAGs, in the interstitial compartment of *Idua-/-*6m mice compared with the *Idua+/+*6m controls ($P < 0.0001$) (**Figure 1H-K**).

We did not observe any statistically significant differences among the *Idua+/+* and *Idua-/-* groups in the diameter of seminiferous tubules and epithelium height. However, the frequency of interstitial area was higher in *Idua-/-*3m ($P = 0.0002$) and *Idua-/-*6m ($P = 0.048$) groups (**Table 2**).

Immunohistochemistry

Cross sections stained with anti-caspase 3 were more intensely labelled in *Idua-/-*3m mice compared with the corresponding controls ($p = 0.04$), but no statistically significant difference was observed between the 6-month-old groups (**Figure 2**). No difference was observed between the *Idua+/+* and *Idua-/-* cross sections stained with anti-bcl-2 (**Figure 2**).

Discussion

Due to the multisystemic nature of MPS I, a set of tissues and organs has been investigated in animal models to elucidate some of the effects of improper GAG storage in different cell types. The reproductive system is usually an important object of investigation in understanding a variety of syndromes, and must also be examined in animal models of LSD, such as MPS.

Furthermore, no study specifically evaluates male or female reproductive parameters in any of animal models of MPSs. Until the present moment, there is a single study related to the frequency of pregnancies of female MPS VII mice submitted to copulation with normal and knockout males. They demonstrated an improvement in sexual behaviour provided by the enzyme replacement therapy [18]. In humans, testicular histopathological signs were detected in a 19-year-old boy autopsy with MPS II [19]. Regarding pregnancies, some cases have been reported in MPS I women submitted to therapeutic interventions [20, 21].

Male reproductive organs, such as the testes, epididymis, ventral prostate and seminal vesicles, require androgens for maintenance throughout life, and testosterone is one of the most important hormones involved in this function [22]. Although the testicular and epididymal biometric parameters were not reduced in the *Idua-/-* groups, the absolute prostate weight in the 3-month-old *Idua-/-* mice and the seminal vesicle weights in both *Idua-/-* groups were decreased compared with the control groups. However, the concentration of plasma testosterone was not statistically different between the *Idua+/+* and *Idua-/-* groups, suggesting that the testosterone biosynthesis at least is not impaired in *Idua-/-* mice. Similar results have been reported in a knockout model of a lysosomal glycoprotein that is associated with LSDs such as Gaucher, Tay Sachs and metachromatic leukodystrophy [23]. All reproductive biometric values were decreased regardless the concentration of plasma testosterone, which was higher than or equal to the levels in the control group. This evidence suggests that storage of substrates may impair the hormonal response that would result in tissue growth. For this reason, further investigations into the tissue androgen concentrations and signalling mechanisms mediated by androgens are important to understand this growth impairment.

Garcia *et al.* [24] have characterised the murine model of MPS II and have demonstrated increases in the relative weight of the kidneys, liver, spleen, brain and even the testes and epididymis in 10-month-old mice. MPS I and MPS II result in the accumulation of dermatan and heparan sulphates and manifest similarly [2, 24]. Organomegalies were frequent in our *Idua-/-* animals, especially in the *Idua-/-*6m group; however we did not visibly note testicular or epi-

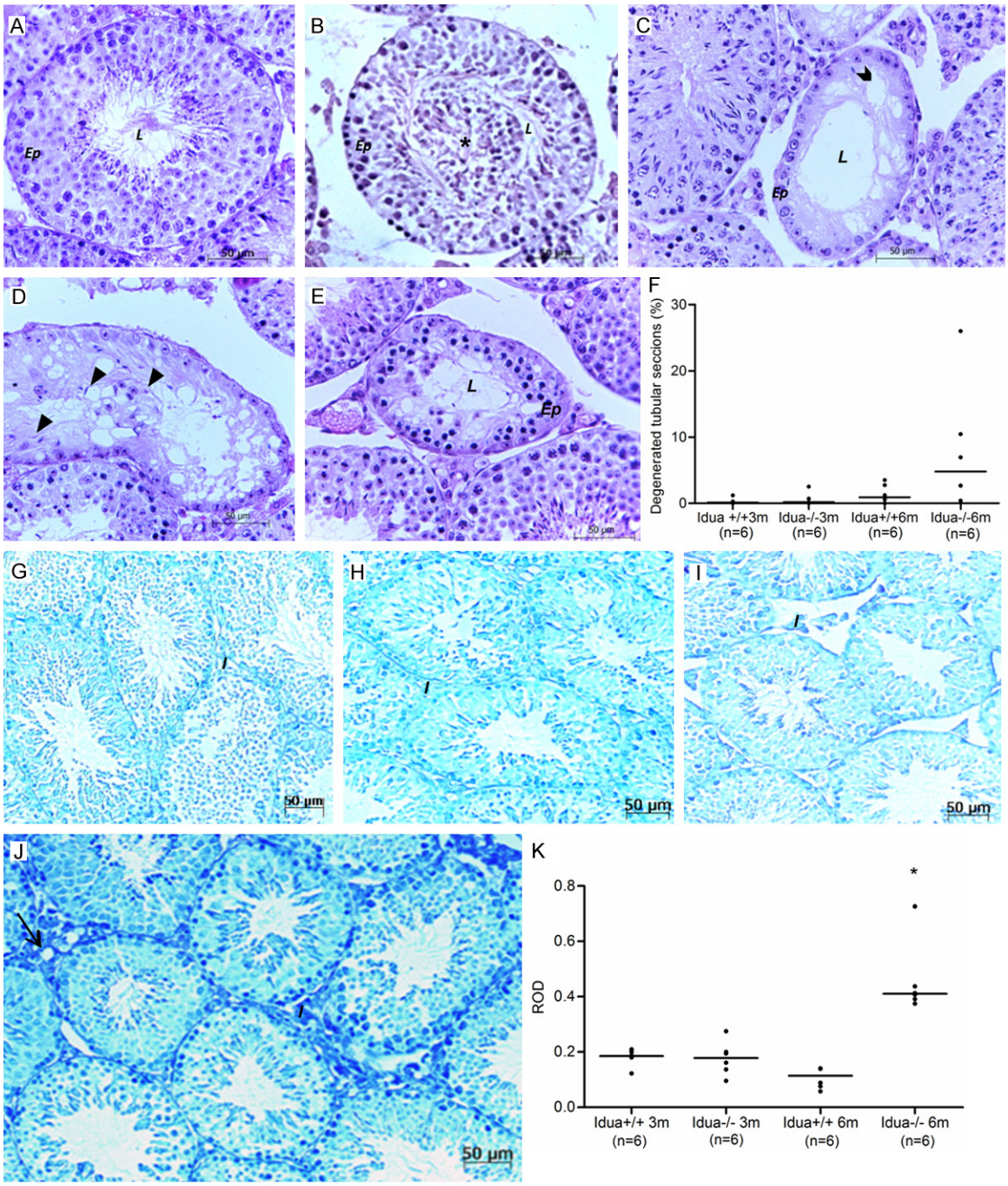

Figure 1. Histopathological evaluation. (A) Normal seminiferous tubule; (B) Tubule with desquamation of immature germ cells in luminal portion (asterisk); (C) Sertoli-cell-only tubule. Note the vacuolisation of Sertoli cell (arrow head) and the absence of germ cells; (D) Signs of necrosis. Note the fragmented or shaded nuclei of germ cells (arrow head); (E) Disorganisation of seminiferous epithelial histoarchitecture with loss of cell layers; (F) Frequency of degenerated tubular sections. Raw data separated by median (bar). Unpaired t-test; (G-J) Toluidine blue stained testicular sections from *Idua+/+*3m (G); *Idua-/-*3m (H); *Idua+/+*6m (I); and *Idua-/-*6m (J). (J) Note the presence of vacuoles (arrow) and the staining intensity in interstitial compartment; (K) Quantitative analysis of the staining intensity with toluidine blue. Unpaired t-test (*$P < 0.0001$), compared to *Idua+/+*6m; n = 6 animals/group. *Ep*: seminiferous epithelium; *I*: interstitial compartment; *L*: lumen. *ROD*: relative optical density. Scale bar = 50 µm. Staining: hematoxylin/eosin (A-E) and toluidine blue (G-J).

didymal organomegaly, as observed in the heart, liver and brain. We only detected an increase in relative testicular weight in the *Idua-/-*3m group, which was not observed in

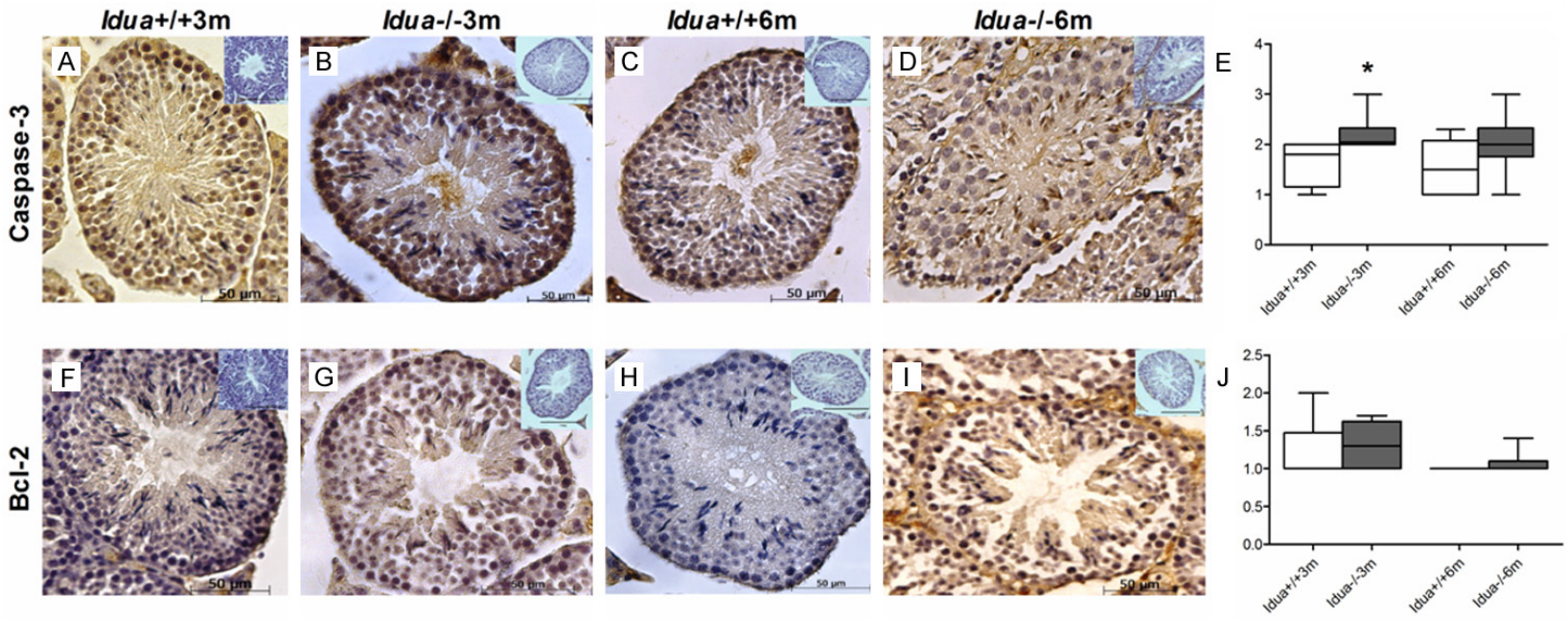

Figure 2. Immunohistochemical analyses. Testicular sections stained with pro-apoptotic caspase-3 (A-D) and anti-apoptotic bcl-2 (F-I) antibodies; (E, J) Quantitative analysis of the intensity of the immunohistochemical reaction. Top right images represent negative controls. Unpaired t-test. *$P \leq 0.05$, compared to *Idua+/*+3m. Scale bars = 50 µm and 100 µm for negative controls.

*Idua-/-*6m mice, possibly as a result of a disproportionate growth between the testes and body during the animal ageing process since testicular absolute weights are similar among *Idua-/-*3m and *Idua-/-*6m and body weight is subtly lower in *Idua-/-*3m group.

Evidence of subfertility and infertility, such as reduced litter size and frequency of pregnancy; have been reported in some studies using animal models of Sandhoff [25], Niemann-Pick [9], Gaucher [7] and Krabbe [26] disorders, which are all categorised as LSDs. Some of the studies have detected a lower affinity for the oocyte pellucid zone and suggested impairments in sperm maturity [7, 9, 26]. In our study, we observed a decrease in DSP that was significant in the *Idua-/-*6m group, although *Idua-/-*3m presented similar values, suggesting impairment in spermatogenesis, which was confirmed by the marked histopathological alterations observed in 6-month-old group.

Histopathological signs, such as interstitial GAG accumulation, Sertoli-cell-only tubules, vacuoles and epithelial desquamation, were expected since heparan sulphate proteoglycans are found in the testicular and epididymal extracellular matrix and are important for signalling during spermatogenesis and sperm maturation [27, 28]. We observed a high variability in the frequency of damaged tubules among the *Idua-/-* animals. However, the tubules from all *Idua+/+* animals were notably healthy with a low frequency of damage.

Six-month-old knockout mice (*Idua-/-*6m) presented vacuoles in the interstitial compartment, which have also been detected in 8-month-old *Idua-/-* mice [29]. Toluidine blue-stained sections suggested deposits of basophilic material in the interstitial compartment of *Idua-/-*6m mice regardless the frequency of degeneration and signs of necrosis, probably as a consequence of progressive GAG accumulation. Toluidine blue staining was more intense especially in *Idua-/-*6m group and the percentage of interstitial area was higher in both *Idua-/-*3m and Idua-/-6m groups, which suggest structural changes in interstitial compartment of seminiferous tubules.

Sperm morphology did not differ between the *Idua+/+* and *Idua-/-* groups, in contrast to the differences observed in other LSD animal models [9, 30-32]. Interestingly, we observed similar percentages of head-lost and tail-lost sperm in the *Idua-/-*3m and *Idua-/-*6m groups, which were significantly higher than *Idua+/+*3m group, suggesting that MPS I may confer age-related sperm fragility. However, we are currently only able to suggest that MPS I interferes with spermatogenesis in a quantitative but not necessarily in a qualitative way.

During spermatogenesis, apoptosis occurs approximately in more than 50% of total germ cells. Sertoli cells are responsible to phagocytosis these apoptotic germ cells and degrade their components by lysosomal enzymes [33, 34]. Some studies have reported associations between increased caspase expression and decreased sperm count, motility, morphology and other andrological pathologies, although apoptosis is required for normal spermatogenesis to eliminate abnormal sperm and to protect heritable genome [35-37]. Interestingly, testicular cross sections were more intensely stained with anti-caspase-3 antibody in the *Idua-/-*3m mice, while staining in the *Idua-/-*6m group was not statistically different from the corresponding control group.

As the lysosomes are intensely required to promote seminiferous tubules clearance, we suggest that the disease progression possibly interfere in the mechanism of cell death control in case of lysosome storage in Sertoli cells.

Our results indicate that the absence of Idua impacts male reproductive parameters, such as the continued growth of seminal vesicles despite normal levels of plasma testosterone, and reduced DSP. In addition, we observed testicular histopathological signs such as interstitial vacuolization and suggest a GAG storage in the same compartment, especially in the 6-month-old mice, indicating disease progression.

The physiological, biometrical and histopathological changes reported in this study are essential for further cellular and molecular experiments aiming to characterise the cellular mechanisms that might be impaired as a consequence of Idua deficiency.

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Disclosure of conflict of interest

None.

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