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Imaging seminiferous tubules – a 9.4T MRI mouse model

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Short title: MRI of mouse testes

20 **Abstract**

21 Fertility problems affect many couples. Research into male fertility commonly uses mouse models
22 due to their availability and similar spermatogenesis to humans. A common target is the seminiferous
23 tubules, the site of spermatozoa production, typically studied using biopsies and histological analysis.
24 High-field Magnetic Resonance (MR) may offer a non-invasive alternative to investigate testicular
25 function in infertility models. Here, we assess seminiferous tubules structure in sacrificed mice to
26 determine the usefulness of MR compared to histology. Twelve mice (11 aged 35-57 days, one >9
27 months) were sacrificed and MR imaged at 9.4T with a Rapid Acquisition with Relaxation
28 Enhancement sequence. Testes were scanned in situ for all mice, and excised in a subset of mice (n=4).
29 A second subset of mice (n=4) had their testes selected for histological analysis. Seminiferous tubule
30 diameter was measured manually from MRI and histology images. Custom image analysis scripts
31 were created for the automated segmentation of seminiferous tubules and calculation of tissue
32 volumes. All ex vivo and in situ images of testes exhibited clear outlines of seminiferous tubules.
33 Ratio of total testis volume to volume of seminiferous tubules did not differ significantly between ex
34 vivo and in situ measurements, and were similar in mature and younger mice. Both total testis volume
35 and seminiferous tubule volume were larger in the mature animal. While histological slices trended
36 towards larger average seminiferous tubules diameter than MRI images, we found no significant
37 differences between MRI and histological measurements. High-field MRI can be used in a mouse
38 model to assess testicular structure in situ. All volumetric measurements compared favourably with
39 histological data. In situ scans also clearly showed identifiable extra-testicular tissues, such as
40 epididymis and prostate tissues. The potential to image tissues associated with sperm maturation as
41 well as spermatogenesis emphasises how MR could be a useful technique in mouse models of fertility,
42 however further work is required to optimize tissue segmentation and validate this method for use in
43 longitudinal studies. This type of measurement could be extended to human fertility studies in the
44 future.

45

46 **Introduction**

47 The production of sperm during the reproductive period in male mammals requires several key steps,
48 many of which are vulnerable to damage. Spermatogenesis is initiated in the epithelium of the
49 seminiferous tubules, which are convoluted, tightly packed tubes that constitute most of the testis
50 volume. Here, germ cells proliferate, go through meiosis and differentiation, before travelling via the
51 rete testis into the epididymis, where they mature and gain the capacity for motility (1). Structure and
52 function of the seminiferous tubules can be damaged by a range of medical conditions and
53 environmental insults, including viral infections (2), diabetes (3), ischaemic insult (4, 5) and exposure
54 to toxic compounds (6-8).

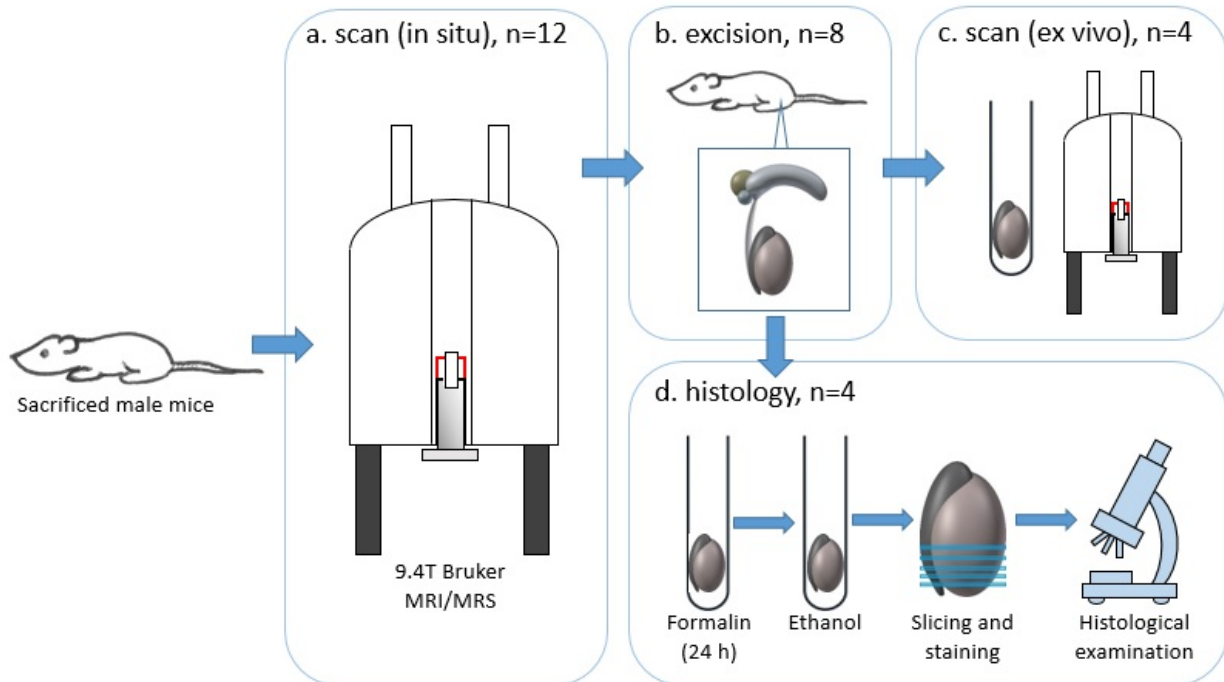
55
56 Mouse models are frequently used in male fertility research, due to their similarities with human
57 spermatogenesis processes (1). Such models can be used to identify causes of defects or manipulate
58 factors influencing key steps in spermatogenesis, which may not be practical nor ethical in human
59 studies. Investigation of testis structure and function in mouse models of fertility typically requires
60 dissection and histological analysis for visualisation and volume measurements of testes and their
61 different sub-structures (e.g. seminiferous tubules, epididymis). These are invasive procedures
62 necessitating the sacrifice of the animal, and are thus not compatible with longitudinal studies.

63
64 High-field magnetic resonance imaging (MRI) is non-invasive and has resolution capable of
65 visualising the internal structures of mice testes. In this study, we investigate how MRI can be used
66 to non-invasively assess testis size and structure in sacrificed mice, focusing on automated
67 identification and segmentation of seminiferous tubules as a measure of testicular development. We
68 seek to compare 9.4T MRI images of the mouse male reproductive system both in situ and ex vivo
69 with histological assessments in the same testes. We will comment upon the usefulness of MRI in
70 murine models of testicular health and disease and the potential for further development of this
71 technique in human clinical investigations.

72 Methods

73 MRI protocol

74 Fig 1 illustrates the study protocol. Twelve sacrificed mice (11 aged 37-57 days, mean 41.6 ± 7.8 , and
75 one mature mouse (>9 months)) from strains with no associated testicular dysfunction were scanned
76 in situ in a 9.4T MR scanner (Bruker BioSpin GmbH, Karlsruhe, Germany). The work was approved
77 by the Project Applications and Amendments Committee (a sub-committee of the University of
78 Sheffield Animal Welfare and Ethical Review Body). The animals were sacrificed using schedule 1
79 methods for reasons not related to this study. T1-weighted Fast Low Angle Shot (FLASH) sequences
80 were obtained to identify the reproductive organs and plan for high-resolution imaging of testicular
81 tissue. High-resolution imaging (in plane resolution 30-90 μ m) was performed with a Rapid
82 Acquisition with Relaxation Enhancement (RARE) sequence (TR/TE 5000/28 ms, see Table 1 for
83 full acquisition parameters). In a subset of mice (n=8), testes were excised. Out of these eight excised
84 testes, four were re-imaged (one fresh and three after fixing in 10% buffered formalin). The other
85 four were selected for histological analysis (see below). All animals and fresh ex vivo tissues were
86 scanned immediately or within 24 hours of sacrifice (storage at -20°C), and fixed tissues were scanned
87 within one week of sacrifice.



88
89 **Fig 1. Schematic of protocol.** Sacrificed male mice were first scanned in situ (a), followed by
90 excision of testes in a subset of mice (b). Testes were then either scanned (c) or prepared for
91 histological examination (d).

92

93 **Table 1: Scan parameters**

| Tissue | N | RARE Factor | TE (ms) | TR (ms) | Slices/ Thickness (mm) | NEX | Matrix | FOV (mm) | Resolution (mm/px) | Scan time (min) |
|---------|-----|-------------|---------|---------|------------------------|-----|---------|-----------|--------------------|-----------------|
| In situ | N01 | 8 | 28 | 5000 | 27/0.25 | 8 | 256/256 | 14.1/17.5 | 0.055 | 42.40 |
| | N02 | 4 | 28 | 5000 | 15/0.5 | 8 | 256/256 | 23/23 | 0.090 | 42.40 |
| | N03 | 4 | 28 | 5000 | 17/0.5 | 8 | 300/300 | 25/25 | 0.083 | 50.00 |
| | N04 | 4 | 28 | 5000 | 19/0.5 | 8 | 300/300 | 25/25 | 0.083 | 50.00 |
| | N05 | 4 | 28 | 5000 | 29/0.5 | 50 | 522/533 | 23.5/24 | 0.045 | 554.10 |
| | N06 | 4 | 28 | 5000 | 37/0.5 | 64 | 533/533 | 24/24 | 0.045 | 709.20 |
| | N07 | 4 | 28 | 5000 | 33/0.5 | 32 | 556/489 | 25/22 | 0.045 | 325.2 |
| | N08 | 4 | 28 | 5000 | 31/0.5 | 64 | 533/489 | 24/22 | 0.045 | 650.4 |
| | N09 | 4 | 28 | 5000 | 21/0.5 | 8 | 500/390 | 22.5/17.5 | 0.045 | 64.4 |
| | N10 | 4 | 28 | 5000 | 21/0.5 | 8 | 507/511 | 22.8/23 | 0.045 | 84.4 |
| | N11 | 4 | 28 | 5000 | 23/1.0 | 8 | 533/523 | 24/23.5 | 0.045 | 86.4 |
| | N12 | 4 | 28 | 5000 | 33/0.5 | 8 | 556/522 | 25/23.5 | 0.045 | 86.4 |
| Ex vivo | N01 | 8 | 28 | 5000 | 19/0.5 | 512 | 316/231 | 7.9/5.8 | 0.030 | 955.4 |
| | N02 | 4 | 28 | 5000 | 19/0.5 | 180 | 356/240 | 10.4/7.3 | 0.030 | 900.0 |
| | N03 | 4 | 28 | 5000 | 27/0.5 | 128 | 512/300 | 15.5/6 | 0.030 | 800.0 |
| | N04 | 4 | 28 | 5000 | 19/1.0 | 128 | 433/300 | 13/9 | 0.030 | 800.0 |

94 N=subject number; RARE= Rapid Acquisition with Relaxation Enhancement; TE=time echo;
 95 TR=time repetition; NEX=number of averages; FOV=field of view.

96

97 **MR image analysis**

98 Slices with clear seminiferous tubules were selected for each animal. Seminiferous tubule diameter
 99 was measured manually (single axis) using Paravision v5.1 (Bruker BioSpin). As tubular diameter is
 100 not uniform, an average of nine tubules across three slices (approximately centre-testis) were
 101 measured and the diameters averaged. Regions of interest (ROIs) were drawn around all slices judged
 102 to contain testicular tissue for one testis, chosen at random, from each animal. A custom MatLab
 103 (Mathworks, Natick, MA, USA) image analysis script was then used for segmentation of
 104 seminiferous tubules and calculation of tissue volumes. The script included three separate steps. First,
 105 full testis tissue volume was calculated based on the sum of all voxels in the ROI. Second,
 106 seminiferous tubules were segmented from testis tissue through thresholding (Otsu method (9)) to
 107 remove background noise. The segmentation was based on standard deviation (SD) of the mean

108 signal within the ROI (low signal threshold: 1.5xSD, high signal threshold: 3xSD; thresholds were
109 derived from preliminary analysis of excised tissue from mature animal). Third, seminiferous tissue
110 volume was calculated based on the sum of the volumes of the non-zero voxels in the segmented
111 images. The script was optimised using scans of excised tissues and subsequently implemented for
112 the in situ scans.

113

114 **Histological preparation**

115 Testes were excised and samples kept in 10% buffered formalin (NBS, Sigma, Irvine, UK) for 24
116 hours. The tissues were then dehydrated in an alcohol series to ensure complete water removal, treated
117 with xylene and embedded in paraffin. Paraffin blocks were cut into 5µm sections and stained with
118 hematoxylin and eosin (H&E, Surgipath Europe LTD, Peterborough, UK). Sections were imaged
119 using 4X and 10X objectives (Olympus CKX41). Nine measurements of seminiferous tubule
120 diameter were made across three sections (taken at approximately 50µm, 300µm and 550 µm from
121 the testis edge) at 10X magnification (cross-sections of tubules) and the mean diameter calculated.

122

123 **Statistical analysis**

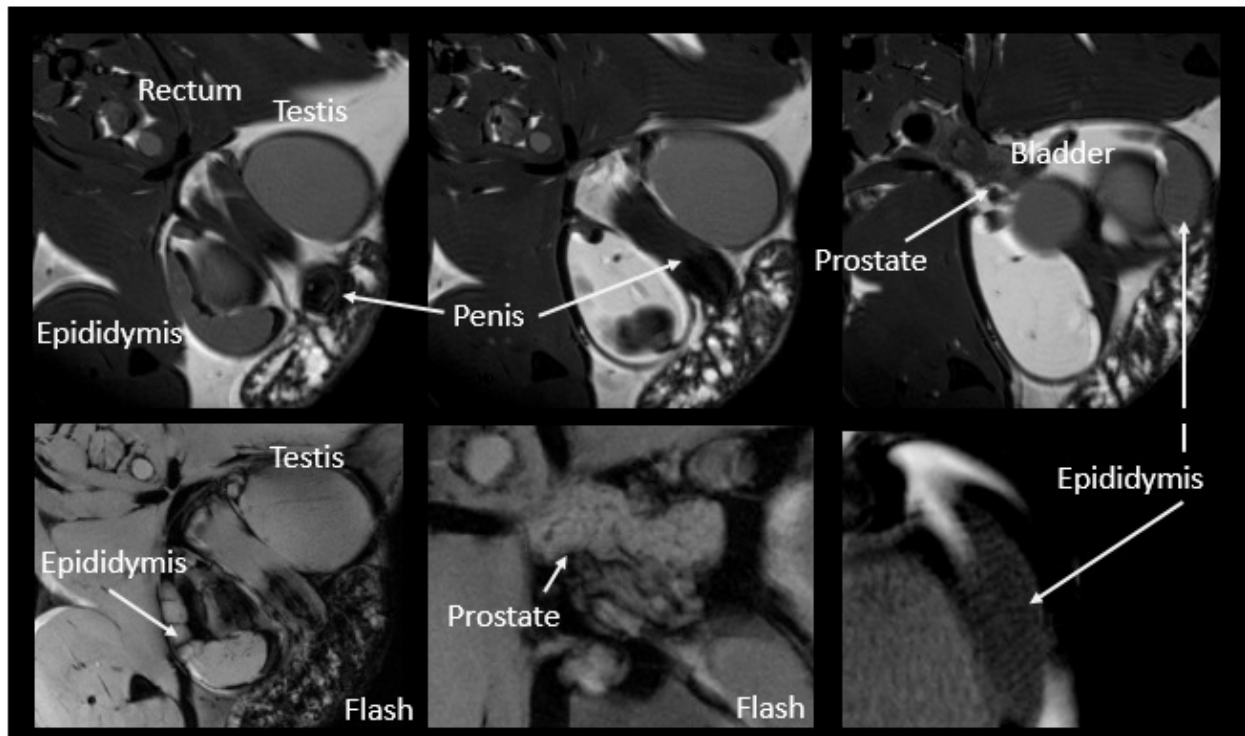
124 Volumetric measurements were compared using a paired Student's t-test, and the relationship between
125 volumes and ages of the animals interrogated using linear correlation (MatLab). All values are quoted
126 as mean ± SD.

127 **Results**

128 **Images**

129 Extra-testicular tissues were observed in situ using both FLASH and RARE imaging sequences and
130 included the epididymis, prostate gland, bladder and penis (Fig 2). All animals yielded usable ex vivo
131 and in situ images of testes, with clear outlines of seminiferous tubules (Fig 3). Histological analysis
132 produced detailed images of testicular tissue with delineated seminiferous tubules (Fig 4). An
133 example of identification of seminiferous tubules using automatic segmentation is shown in Fig 5.

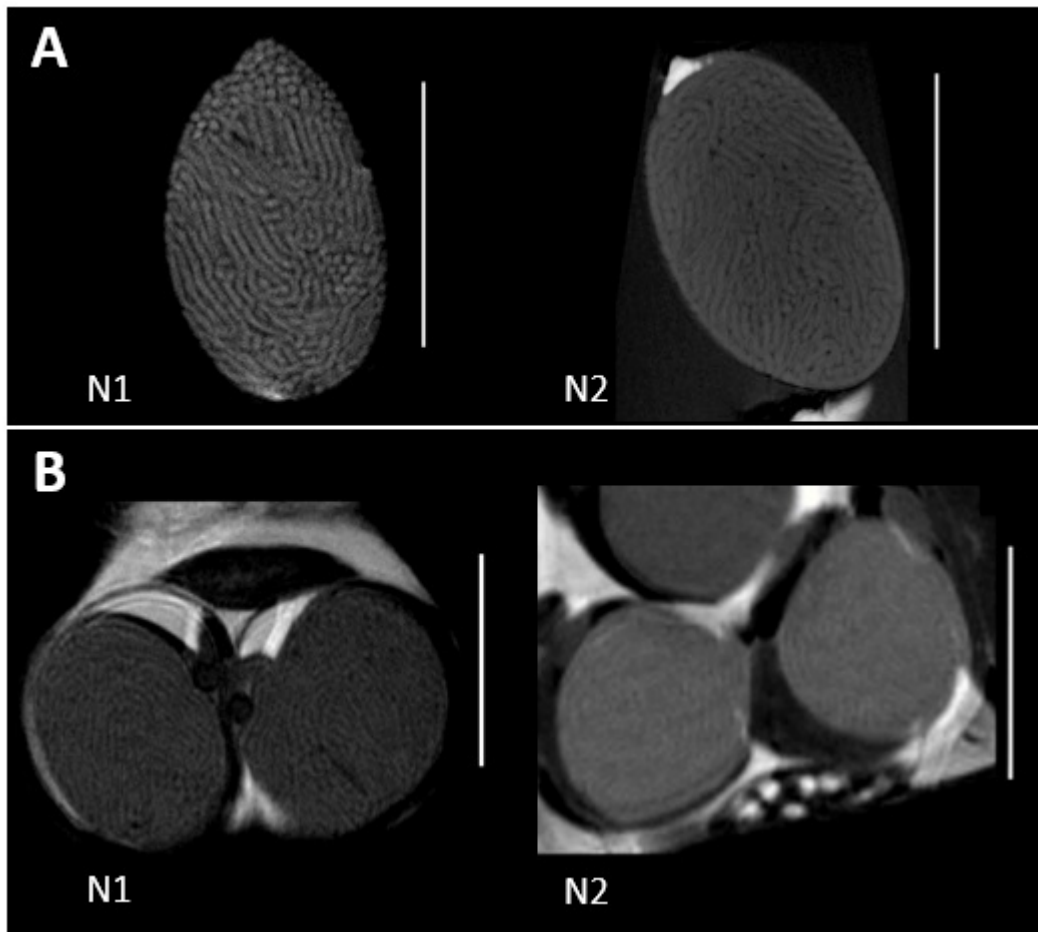
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136 **Fig 2. Testes and extra-testicular tissues obtained in situ.** Images are from subject N5 and
137 representative for the sample. Scan details are presented in Table 1. Images denoted 'Flash'
138 were acquired using a T1-weighted Fast Low Angle Shot (FLASH) sequence (TE=8ms, TR=500ms, 32
139 averages, FOV=23.5x24mm, matrix=522/533).

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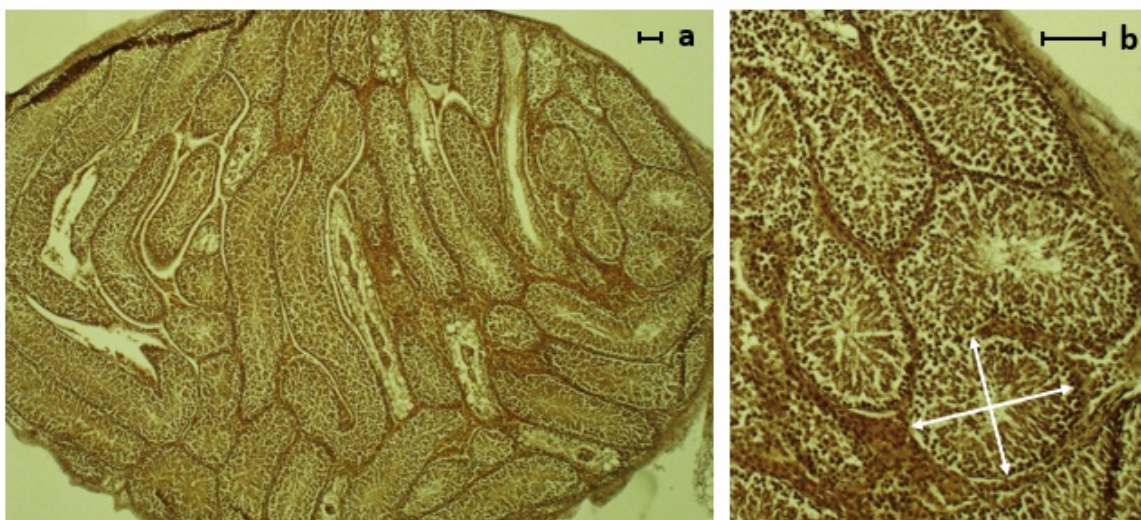
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Fig 3. Structural MRI of mouse testes, ex vivo (A) and in situ (B). Slices approximately center-testis. Images are representative of the sample and include the mature reference individual (N1) and a younger animal (N2, age 35 days). Vertical white bars represent 5mm. Scan details are presented in Table 1.



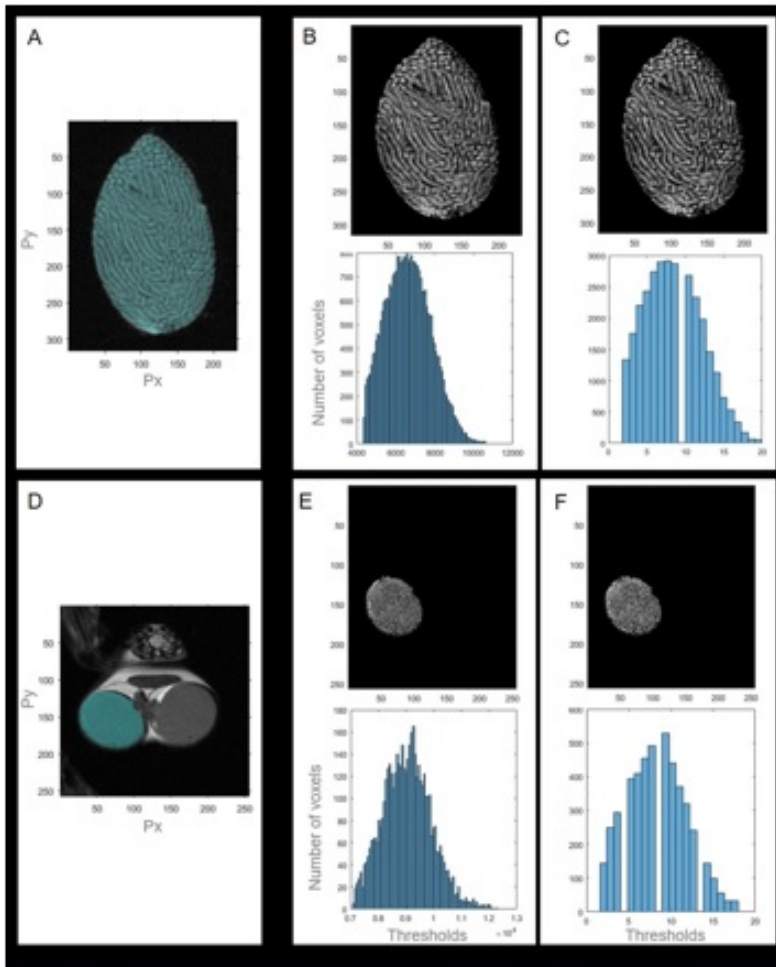
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Fig 4. H&E staining of testes. Images are from subject N11 and representative for the sample. a=4x magnification, b=10x magnification. Scale bars (right corners) both 100µm. White arrows showing example of cross section used for measurements (10).



150

151 **Fig 5. Example of identification of seminiferous tubules using segmentation in subject N1.**

152 Regions of interest were drawn around testis tissue (A and D). Images were thresholded (B and E)

153 using the Otsu method in MatLab, with high and low thresholds assigned based on standard deviation

154 of the mean signal (low 1.5xSD, high 3xSD). Seminiferous tubules and total tissue volume was

155 determined based on the sum of voxels in the processed images. Histogram represents number of

156 voxels at the threshold level used. A-C, ex vivo; D-F, in situ. C and F, stratified images.

157

158 **Calculated volumes**

159 Volumes were calculated for in situ (n=12) and ex vivo scans (n=4). In situ scans showed a total testis

160 volume of $63.2 \pm 7.6\mu\text{l}$ (mean \pm SD), a seminiferous tubules volume of $57.0 \pm 6.9\mu\text{l}$, and a

161 seminiferous tubule volume to total testis volume ratio of 0.90 ± 0.01 .

162

163 Comparing the scans in mice selected for ex vivo MRI (n=4, one fresh and three fixed in 10% buffered

164 formalin), we observed no significant differences between in situ and ex vivo measurements. In these

165 animals, the total testis volume was $69.9 \pm 5.5\mu\text{l}$ in situ and $69.9 \pm 2.2\mu\text{l}$ ex vivo (p=0.97), the

166 seminiferous tubules volume was $63.1 \pm 5.1\mu\text{l}$ in situ and $63.2 \pm 1.7\mu\text{l}$ ex vivo (p=0.99). One animal

167 (N3) showed lower volume (but no change in volume ratio) ex vivo due to compressed tissues in the
168 sample tube and was excluded from the comparison. Ratio calculations showed no difference in situ
169 versus ex vivo (both 0.90 ± 0.01 , $p=0.94$).

170

171 **Seminiferous tubules diameter**

172 Average seminiferous tubule diameter measured from in situ RARE images was $164 \pm 16\mu\text{m}$ (range
173 141-198, $n=12$). Comparing measurements of seminiferous tubule diameter in the mice selected for
174 histological analysis ($n=4$), we observed no significant differences between averages obtained using
175 histology and using MRI. MRI analysis in this subset showed average tubule diameter of $156 \pm 7\mu\text{m}$
176 (range 147-163, in situ images). Histological analysis in the same animals showed larger diameters
177 ($169 \pm 7\mu\text{m}$, range 160-175), but this difference did not reach statistical significance ($p=0.11$).

178

179 **Age effects**

180 Total testis and duct volume was larger in the mature reference animal compared to the mean of the
181 younger individuals by 19.7% and 19.8% respectively (no statistical comparisons). Calculated total
182 testis volume to seminiferous tubules ratio was the same in the mature animal as younger mice, but
183 the mean width of the seminiferous tubules were larger size in the mature animal (12.2%, no statistical
184 comparisons). There were no significant correlations between age and testes or seminiferous
185 volumetric measurements within the main sample (aged 37-57 days).

186 **Discussion**

187 **Key findings**

188 Mouse seminiferous tubules structure and extra-testicular tissues may be visualised in situ using 9.4T
189 MRI. MR images may be analysed and volumetric information quantified using automated image
190 analysis scripts.

191

192 **Quantification of images**

193 The automated image analysis yielded data compatible with previous histological findings in similar
194 animal populations (10-12). We observed that total testis size was greater in the mature individual
195 compared to younger mice (10), but there was predictably no significant correlation with age in the
196 main sample, in which the majority of the mice were of a similar age (35-38 days old, N=7). The
197 volume of seminiferous tubules was matched to testicular volume, as the total testicular volume to
198 tubules ratio was similar between individuals irrespective of testes size. This suggests no change in
199 testicular architecture within the age group (10). In the subset of mice selected for ex vivo imaging,
200 the calculated volumes did not differ between ex vivo and in situ scans. Our quantification method
201 thus yields repeatable and reliable volume estimates. Furthermore, as in situ scans were collected
202 over a shorter period and with fewer averages than ex vivo scans (Table 1), this indicates that our
203 analysis method remains robust across different levels of contrast.

204

205 We obtained in situ scans with in-plane resolution of 45 μ m (slice thickness 0.5mm), which allowed
206 us to observe the tubules (typically 150-200 μ m in diameter). Manual estimates of tubule diameter
207 were not significantly different to, albeit trending towards lower values than, those observed using
208 histology. Lower resolution and partial volume effects in the MR images may account in part for this
209 trend. Additionally, as thresholding was used to segment seminiferous tubules to obtain a clearer view
210 of the testicular composition, this could cause edges of tubules to be consistently underestimated as
211 a single voxel width was used to delineate adjacent tubules. Interpolating the images may improve
212 the accuracy of diameter estimation. While histology remains the gold standard for measuring
213 absolute tissue morphometry in small structures, MRI might be more accurate in larger structures as
214 it circumvents sources of tissue distortion (e.g. in murine brain imaging (13)).

215

216 MRI has a wider Field of View capability than most invasive procedures (e.g. histological analysis),
217 permitting the 3D exploration of whole structures with excellent soft tissue contrast. Comparatively,
218 histological analysis is limited to selected, small slices. MRI by contrast can capture full 3D images
219 of structures that would otherwise be too large for standard histological analysis. Here, we reliably

220 obtained clear structural images of urogenital components, including the epididymis, penis, prostate
221 and bladder (Fig 3). The epididymis is of particular interest for fertility research, as sperm maturation
222 and development of motility potential occurs in this tissue (14). This unique temporal and spatial
223 capability makes MRI a valuable tool, and it has already been used in a range of murine models of
224 human disease (13, 15, 16) and physiology (17-19). Yet it remains underutilised in fertility research.

225

226 At 9.4T it is possible to obtain greater resolution and contrast than those obtained here, although this
227 comes at the cost of extended scan time. Fig 3 shows *ex vivo* and *in situ* images of testes from two
228 animals, illustrating the added contrast obtained in longer scans (*ex vivo*) compared to shorter scans
229 (*in situ*). Despite this difference, our automated volume calculations were highly similar for *in situ*
230 and *ex vivo* scans. Furthermore, T2 weighted fast spin-echo images with resolution of 30 μ m has been
231 achieved in 60 minutes for murine *in vivo* experiments without contrast agents (20). In rat testes, *in*
232 *vivo* scans at lower field strengths (4.7T) show tubular structure in T2 weighted images with scan
233 times of 68 minutes or less (5). Even lower field strengths (1.5T) have successfully been used in rats
234 to detect hypoperfusion following experimental testicular torsion using scan sequences lasting less
235 than 10 minutes (5, 21). This, combined with our high-resolution structural images *in situ*, strongly
236 suggests that high field strength MRI is both feasible and useful when investigating murine testes *in*
237 *vivo*.

238

239 **MRI in murine models of fertility**

240 Translation from animal research to human medicine is greatly aided by longitudinal experimental
241 designs. Such protocols add a temporal component to pre-clinical research that is generally
242 considered central to fully characterise physiological processes. MRI is uniquely positioned to
243 conduct this type of research as its non-invasive nature permits the study of progressions of disease
244 or development within-subject, resulting in increased experimental power and the potential for using
245 (and sacrificing) fewer animals. This is in line with the principle of the 3Rs in animal research:
246 Replacement, Reduction and Refinement.

247

248 The potential of MR extends beyond structural imaging, as live mice offer a range of additional
249 functional data. Diffusion imaging or functional imaging may provide information on fluid movement
250 and blood perfusion. Regional ischaemia, heightened perfusion, bloating vessels, and blocked or
251 damaged seminiferous tubules can all be imaged at high resolution. Contrast agents may be employed
252 to further optimise contrast and track vascularisation. Magnetic resonance spectroscopy (MRS)
253 information can also be obtained. MRS is a highly powerful tool for metabolic research that may

254 allow us to probe spatially discreet metabolism. For example, MRS might be used to investigate
255 testicular tumour metabolism in vivo, or to track spermatogenesis variation over time within
256 seminiferous tubules.

257

258 **Clinical potential**

259 MRI/MRS may also be valuable clinical tools. For example, MRI can be used in humans to visualise
260 intratesticular lesions (22-24), testicular tumours (25), torsion (21, 26), varicoceles (27, 28) and
261 testicle location (29) at common clinical MR field strengths. Reduction in testicular size, epididymis
262 diameter or seminiferous tubule diameter (30) may be markers of disrupted spermatogenesis, and the
263 size of either could thus potentially be used as an indicator of pathology. This is supported by evidence
264 from MR images in rats with chronic spermatogenic impairment (5). MRI assessments may also be
265 used to guide testicular biopsies (31), reducing the number of invasive events and thus lessen the
266 chance of surgical complications and infertility (32). Furthermore, recent findings suggest that MRS
267 may be used to assess metabolite concentration in the testes in both humans (33) and rats (34), which
268 could offer information on spermatogenesis (33), possibly through assessment of phosphocholine
269 concentration as suggested by ¹H magnetic resonance spectroscopy of snap frozen testicular tissue
270 from men with azoospermia (35). MR could thus be a useful technique in the clinic as well as a
271 valuable tool for experimental models of fertility.

272

273 **Limitations**

274 The present study was conducted in deceased animals, and tissues were thus ischaemic at the point
275 of scanning. Some tissue degradation and blood pooling may also have occurred despite scanning
276 commencing immediately after sacrifice. Both ischaemia and tissue degradation would contribute to
277 reduced image quality.

278

279 **Conclusions**

280 Damage to the testes may significantly impair fertility, and the investigation of testis structure is
281 central to research in male fertility. Short breeding cycles along with comparable spermatogenesis to
282 humans and a range of relevant breeds make the mouse model highly useful. High-field MRI is a
283 useful tool for mouse models of testicular morphology. Testicular composition can be visualised, and
284 volumetric analysis of MR images is predictable and reproducible for a murine model exhibiting no
285 testicular dysmorphism. The potential to image tissues associated with sperm maturation as well as
286 spermatogenesis emphasizes how MR could be a useful technique in mouse models of fertility.

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291

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