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Development of an acute ovine model of polycystic ovaries to assess the effect of ovarian denervation

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28 **Abstract**

29 Polycystic ovary syndrome (PCOS) seems to be associated with increased ovarian
30 sympathetic nerve activity and in rodent models of PCOS reducing the sympathetic drive to
31 the ovary, through denervation or neuromodulation, improves ovulation rate. We
32 hypothesised that sympathetic nerves work with gonadotropins to promote development
33 and survival of small antral follicles to develop a polycystic ovary phenotype. Using a
34 clinically realistic ovine model we showed a rich sympathetic innervation to the normal
35 ovary and reinnervation after ovarian transplantation. Using needlepoint diathermy to the
36 nerve plexus in the ovarian vascular pedicle we were able to denervate the ovary resulting
37 in reduced intraovarian noradrenaline and tyrosine hydroxylase immunostained
38 sympathetic nerves. We developed an acute polycystic ovary (PCO) model using
39 gonadotrophin releasing hormone (GnRH) agonist followed infusion of follicle stimulating
40 hormone (FSH) with increased pulsatile luteinising hormone (LH). This resulted in increased
41 numbers of smaller antral follicles in the ovary when compared to FSH infusion suggesting a
42 polycystic ovary. Denervation had no effect of the survival or numbers of follicles in the
43 acute PCO model and did not impact on ovulation, follicular and luteal hormone profiles in a
44 normal cycle. Although the ovary is richly inervated we did not find evidence for a role of
45 sympathetic nerves in ovarian function or small follicle growth and survival.

46

47

48 **Key words**

49 Follicle, tyrosine hydroxylase, polycystic ovary, sympathetic nerve, gonadotrophin

50

51 **Introduction**

52 Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting 7-8% of women
53 of reproductive age (1). Although there are metabolic aspects of PCOS, ovarian structure
54 and function are key to its diagnosis (2). It is associated with ovarian dysfunction manifested
55 by anovulation, irregular menstrual cycles, increased thecal androgen synthesis and
56 secretion (2), and multiple non-growing, but functional, antral follicles, giving the classic
57 polycystic ovary morphology (3). Ovarian function is regulated by a combination of systemic
58 gonadotrophins and local growth factors (4). Women with PCOS tend to have relatively
59 higher circulating luteinising hormone (LH) concentrations (2) and altered ovarian growth
60 factor profiles (5).

61 Sympathetic nerves are present within the ovary but the role of ovarian sympathetic
62 innervation remains unclear (6). It has previously been hypothesised that sympathetic
63 nerves act in concert with gonadotrophins to facilitate follicular development and function,
64 including enhancing thecal androgen secretion (7,8). In rodents hyperstimulation of the
65 ovarian sympathetic nerves increases ovarian noradrenaline concentrations and is
66 associated with a polycystic ovary (PCO) phenotype (9). In a rodent model of PCO induced
67 by juvenile exposure to estradiol valerate (EV) there is evidence for increased ovarian
68 sympathetic activity (10). In the rodent EV PCO model surgical denervation of the superior
69 ovarian nerve decreases ovarian noradrenaline concentrations and induces increased
70 ovulation rate (11,12). This suggests the sympathetic nervous system may be a therapeutic
71 target in PCOS (13).

72 There is evidence of an increased sympathetic tone in women with PCOS (14). Women with
73 PCOS have increased muscle sympathetic nerve activity (15) and increased sympathetic
74 drive to the heart (16) and brain (17). Renal sympathetic denervation reduced muscle

75 sympathetic drive as well as improving blood pressure and insulin sensitivity in women with
76 PCOS (18). There is no evidence in women with PCOS whether there is increased
77 sympathetic drive to the ovary and whether ovarian NA concentrations contribute to the
78 development of a PCO morphology.
79 We hypothesised that the sympathetic nervous system works in parallel with
80 gonadotrophins to facilitate the development of a PCO and that ovarian denervation would
81 improve the polycystic ovarian phenotype. Herein we assessed the effect of ovarian
82 denervation in a large animal model that, unlike rodents, has very similar ovarian function
83 to women. We then developed and validated an acute model of PCO using gonadotrophin
84 manipulation and assessed the effect of denervation on gonadotrophin-driven follicle
85 growth.
86
87
88 **Materials and Methods**
89
90 *Animals*
91 We studied adult Scottish Greyface ewes (*Ovis aries*) weighing 60-75 kg in their third to fifth
92 breeding season. Ewes were housed together in spacious pens with ad libitum hay
93 supplemented with Excel ewe nuts (0.5-1.0kg/day; Carrs Billington, Lancashire, UK) and
94 Crystalayx extra high energy lick (Caltech Solway Mills, Cumbria, UK). All experiments were
95 conducted under Project Licence (PPL60/4401; PCD686E93) from the UK Home Office and
96 underwent institutional ethics review. This work was conducted in accordance with Animals
97 (Scientific Procedures) Act 1986, Galvani Policy on the Care, Welfare and Treatment of
98 Animals Policy 040 and approved by the Galvani Bioelectronics Animal Scientific Review

99 Committee and the GSK Policy on the Care, Welfare and Treatment of Animals. Ovarian
100 sections from an earlier study collected 11 months after whole ovarian cryopreservation
101 and transplantation using Scottish Greyface ewes as described in detail previously (19) were
102 available for analysis.
103
104 *Tissue collection*
105 Ewes were killed using a schedule 1 method and ovaries and ovarian vascular pedicles were
106 collected. The vascular pedicles were fixed in Bouin's solution for 24 hours and transferred
107 to 70% ethanol for subsequent paraffin wax embedding. The ovaries were either: 1) fixed in
108 Bouin's solution for 24 hours and embedded in paraffin wax for subsequent
109 immunohistochemistry, 2) halved longitudinally and one half fixed in Bouin's solution and
110 embedded into paraffin wax and the other half snap frozen and stored at -80 ^{o}C for
111 subsequent RNA extraction and measurement of intraovarian noradrenaline or 3) fixed in
112 4% paraformaldehyde for optical projection tomography, depending on the experiment.
113
114 *Immunohistochemistry*
115 Mid-ovarian tissue sections cut to 5µm were mounted on permafrost slides. Sections were
116 dewaxed, rehydrated as described previously (20). Antigen retrieval was carried out by
117 pressure cooking for 5 min in 0.01 M citrate buffer, pH 6.0. Sections were washed in water
118 before peroxidase quenching and blocking steps were performed via incubation with 3%
119 H_2O_2 for 10 minutes, blocking with avidin and biotin (Vector Laboratories Ltd.,
120 Peterborough, UK) and then serum blocking with 20% normal goat serum/ 5% bovine serum
121 albumin (BSA) in Tris Buffered saline (TBS, 0.05 M Tris pH 7.4, 0.85% NaCl). Slides were

122 washed in TBS between treatments, then in TBS containing 0.025% Triton X-100 (TBS-T)

123 prior to serum block and antibody incubation.

124 The primary antibody diluted in serum block (mouse anti-tyrosine hydroxylase 1:1000

125 (Sigma-Aldrich Ltd, Dorset, UK), mouse monoclonal anti-Ki67 1:100 (Novocastra, Newcastle,

126 UK) (1:1000) or rabbit polyclonal anti-caspase 3 1:100 (Cell signalling, MA, USA)) was applied

127 to sections and incubated overnight at 4°C. Slides were washed in TBS-T and the secondary

128 antibody (biotinylated goat anti-mouse or goat anti-rabbit (Vector Laboratories,

129 Peterborough, UK) diluted 1:500 in serum block) was applied to slides for 1 hour. Slides

130 were washed in TBS-T followed by Vectastain ABC Elite tertiary complex (PK-1600 series;

131 Vector Laboratories) for 1 hour after which 3,3′-diaminobenzidine (Dako, Cambridge, UK)

132 was applied for 3 minutes to visualise binding. Sections were then counterstained with

133 haematoxylin and mounted using Pertex mounting medium (Cellpath, Newtown, Powys,

134 UK). Negative controls were non-specific mouse or rabbit serum of equivalent

135 immunoglobulin concentrations in place of the primary antibody.

136

137 *Immunofluorescence*

138 Dual labelled tissue sections were prepared for confocal microscopy following the

139 immunohistochemistry protocol described above, with the following adjustments. The

140 peroxidase wash step was omitted and the slides were permeabilised normally through a

141 series of two five-minute washes in TBS-T. After an incubation time of one hour in 10%

142 normal goat serum, the endothelial antibody (Rabbit monoclonal anti-CD31, Vector

143 Laboratories) and mouse anti-tyrosine hydroxylase antibody were diluted together at a

144 concentration of 1:100 and 1:200 respectively in TBS, before being added to the slides and

145 incubated overnight at 4°C.

146 After washing in TBS with 0.01 % Tween 20 (Sigma-Aldrich, UK) the slides were
147 incubated with biotinylated goat-anti-rabbit secondary antibody diluted in 10 % NGS at a
148 concentration of 1:500 for one hour at room temperature. After washing in TBS-Tween 20
149 (0.01 %) the slides were incubated with Dylight® 594 (Thermo-Fisher, UK) and goat-anti-
150 mouse IgG secondary antibody conjugated to Alexaflour® 488 (Invitrogen, UK), each at
151 1:100 in 10% NGS. Slides were incubated in the dark at room temperature for two hours in a
152 humidity chamber. After washing with TBS-Tween 20 in the dark slides were mounted in an
153 aqueous solution containing 4'6-diamidino-2-phenyllindole (DAPI), and stored for 12 hours
154 at 4°C, prior to visulisation on a Zeiss LSM 880 AxioObserver Z1 confocal fluorescent
155 microscope (wavelengths: 405 nm, 488 nm, 594 nm, laser power set at 2%).
156
157 *Analysis of Tissue Sections*
158 Two examiners, blinded to treatment, graded the immunohistological staining (based on
159 area of staining) of tyrosine hydroxylase independently and the scores were averaged for
160 whole ovary sections. Each ovary section was examined and graded out of four, with zero
161 indicating no staining present and four indicating abundant staining throughout the tissue.
162 Spatiotemporal examinations of vessel and nerve relationships were made using a Zeiss
163 LSM880 Confocal Microscope. Images were captured to illustrate this at 20x magnification,
164 and 63x magnification with oil.
165 Two independent examiners, blinded to treatment, also independently counted the
166 number of follicles from a standardised mid-section of the ovary (21) as well as the number
167 of preantral follicles. Follicles were classified as preantral if they did not show any antral
168 cavity and antral if they showed a clear fluid-filled antrum (>500 µm). Immunohistochemical
169 staining of whole ovary sections stained for proliferation (Ki67) and atresia (activated

170 caspase 3) were blindly examined by two independent expert examiners. Each antral follicle
171 was examined and staining was divided into two classifications, positive (clearly positive
172 immunostaining present in multiple cells) and negative (scant/ absent immunopositive
173 cells). Number of follicles per classification was used for proportional analysis as described
174 previously (21).

175

176 *Ovarian nerve ablation*

177 A mini-laparotomy was performed with sterile technique under general anaesthesia,
178 induced using isoflourane (Isoflo, Abbott Animal Health, Maidenhead, UK). A small
179 paramedian incision exposed the ovaries. To avoid non-specific ovarian damage we
180 specifically targeted the nerves in the ovarian neurovascular pedicle. Needlepoint diathermy
181 using monopolar coagulation current (Surgitran, STW-100) (22) was used to coagulate
182 around the ovarian vessels, in the regions where sympathetic nerves had been identified,
183 leaving blood vessel integrity intact.

184

185 *Intraovarian noradrenaline measurements*

186 A 3 mm^3 sample from the ovarian cortex at the lateral edge of the ovary was used to
187 measure intraovarian noradrenaline (NA) concentrations. It was weighed and homogenised
188 in lysis buffer (0.01N HCl, 1 mM EDTA, 4mM $Na_2S_2O_5$). Lysate was spun at 5000 rpm at 4 ^oC
189 for 10 min and the supernatant analysed. Quantification was carried out using the
190 competitive NA ELISA kit (IMMUSMOL, Pessac, France) as described previously (23)
191 following the manufacturer's instructions. NA was extracted using a cis-diol-specific affinity
192 gel, acylated and then derivatised enzymatically. The antibody bound to the solid phase was
193 detected using an anti-rabbit IgG-peroxidase conjugate and tetramethylbenzidine (TMB) as

194 a substrate. The reaction is monitored at 450 nm. The sensitivity was 2 pg/ml, and the intra

195 and interassay CVs were <9%. The cross reactivity found was 0.14% for adrenaline and 1.8%

196 for dopamine.

197

198 *Plasma hormone measurements*

199 Plasma estradiol and progesterone concentrations were measured using a commercial ELISA

200 following the manufacturer's instructions on a Cobas E411 immunoanalyser (Roche,

201 Mannheim, Germany). The progesterone assay (Cobas progesterone II) has a sensitivity of

202 0.48 nmol/l. The cross reactivity with related steroids is <1%. The estradiol assay (Cobas

203 Estradiol III) has a sensitivity of 11 pmol/l. Apart from 6α-OH estradiol the cross reactivity of

204 related steroids is <1%. Both assays have CVs <10%.

205

206 *Quantitative Real Time (qRT) PCR*

207 RNA was extracted from tissue using RNeasy mini spin columns following manufacturer's

208 protocol and concentration measured using NanoDrop 1000 Spectrophotometer as

209 described previously (20). Complimentary DNA (cDNA) was synthesised from 200 ng RNA in

210 accordance with manufacturer's protocol (Applied Biosystems, California, USA).

211 Subsequently, qRT-PCR was performed using SYBR Green. Real-time PCR reactions were

212 carried out in duplicate 10 µl reactions, negative controls consisted of cDNA reaction

213 without reverse transcriptase and a reaction replacing cDNA with nuclease-free water. Melt

214 curve analysis revealed a single amplicon in all cases. GAPDH has been reported as a suitable

215 internal control for ovarian stromal gene expression (24) and target gene expression was

216 analysed relative to GAPDH and quantified using the DCt method.

217 Primer3 Input version 0.4, online software, was used to design forward and reverse
218 primers from DNA sequences obtained from Ensembl Genome Browser. Sequences were
219 checked for specificity using Basic Local Alignment Search Tool and validity confirmed as
220 previously described (25). The primers 5'-3' were: CCN2: TGCCCTCGCAGCTTACC and
221 CTTGGAACAGGCACTCCACT; VEGF: TCTTCAAGCCATCCTGTGTG and
222 TGCATTCACATTTGTTGTGC; NGF: CTGGCCACACTAAGGTGCATA and
223 GCTGCCTGTATGCCGATCAA; IGF1: CATCCTCCTCGCATCTCTTC and CTCCAGCCTCCTCAGATCAC;
224 FGF2: ACTTTAAGGACCCCAAGCGG and AGTTTGATGTGAGGGTCGCT; GAPDH:
225 GGCGTGAACCACGAGAAGTATAA and AAGCAGGGATGATGTTCTGG.
226
227 *Development of acute model of PCOS*
228
229 Intravaginal progestogen-impregnated sponges (60 mg medroxyprogesterone acetate per
230 sponge; Intervet Laboratories Ltd, Cambridge, UK) were inserted into ewes (n=6) and then 3
231 days later gonadotrophin releasing hormone implants (GnRH; Suprelorin; 4.7 mg Deslorelin
232 acetate; Virbac, UK) were inserted, through large bore needles, for pituitary suppression.
233 Eleven days later the sponge was removed and the sheep were given an injection of
234 prostaglandin F2α (PG; 100 mg Cloprostenol; Estrumate; Coopers Animal Health Ltd, Crewe,
235 Cheshire, UK) to ensure luteolysis of any residual corpora lutea and prepare for an artificial
236 follicular phase. Three days later the jugular vein of the sheep was canulated and infusions
237 started. FSH (Folltropin; Vetoquinol UK Ltd, Buckinghamshire, UK) given at 1mg/hour via
238 jugular catheter using Graseby MS 16A syringe drivers; Luteinizing Hormone (LH; ovine LH
239 NIADDK-oLH-27; Dr. A.F. Parlow, Harbor-UCLA-Medical Center, Torrance, CA) given as 4
240 hourly pulses via the jugular catheter (18 µg/pulse) using Zyklomat pulse infusion pumps, for
241 6 days. Sheep were given either physiological FSH concentrations only with baseline

242 endogenous LH (n=3) or physiological FSH + additional exogenous LH infusions (n=3) (Figure
243 1A). At end of infusions the left ovary from each animal was processed for optical projection
244 tomography (OPT) scanning as described above.
245
246 *Testing the effect of denervation of acute model of PCOS*
247
248 Progesterone sponges were inserted in ewes (n=6) and 7 days later GnRH implants were
249 inserted as described above. Five days later mini-laparotomy was performed as described
250 above followed by unilateral diathermy needle denervation to the left ovary was performed,
251 allowing the right ovary to serve as an internal control. Six days later sponges were removed
252 and PG injections were given as described above. Sheep were cannulated 3 days later and
253 infusions started as described above with FSH (1 mg/hour) (n=3) or FSH+LH (FSH 1 mg/hour;
254 LH 4 hourly pulses of 18 µg/pulse) (n=3) for 6 days (Figure 1B). At the end of infusions both
255 ovaries were processed for OPT scanning as described above.
256
257 *Optical Projection Tomography*
258 Ovaries processed for OPT were fixed in 4% paraformaldehyde overnight, washed 4 x 30
259 minutes Phosphate Buffered Saline (PBS), then 30 minutes each in 30%, 70%, 90%, 100%
260 Ethanol. They were transferred to Methanol for 2 hours, then into fresh Methanol and
261 stored at 4°C until processed for scanning. Ovaries were attached to mounting blocks, and
262 then immersed in BABB (2 parts Benzyl Benzoate, 1 part Benzyl Alcohol) until cleared
263 sufficiently for scanning. Cleared ovaries were scanned in a calibrated Bioptonics 3001
264 tomograph (Bioptonics, UK). Dataviewer (Version 1.5.2.4 Release, July 2015) was used to
265 combine the scans into 2D and 3D models to then quantify the size and number of each

266 follicle throughout each ovary. After scanning ovaries were returned to methanol to remove
267 BABB, then washed 30 min each in 100%, 90% and 70% Ethanol. They were stored in 70 %
268 Ethanol prior to embedding in paraffin wax for sectioning.
269
270 *Statistical analysis*
271 Statistical analysis was conducted using GraphPad Prism 8.0 (GraphPad Software, San Diego,
272 CA) with $P<0.05$ considered statistically significant. Proportional contingency table analysis
273 was measured by Fisher's exact test for 2x2 tables and Chi squared for larger tables. After
274 checking for normality and similar variances two column comparisons were examined using
275 unpaired two-tailed t-tests if the data was parametric and Mann Whitney U tests if not
276 parametric or normally distributed. Correlation was assessed using Spearman co-efficient of
277 correlation.
278
279
280 **Results**
281
282 *Sympathetic innervation of the ovine ovary*
283
284 The ovaries and ovarian pedicle of Scottish Greyface sheep (n=6) were examined
285 macroscopically (Fig. 2A). The vascular bundle, consisting of the ovarian artery closely
286 intertwined with the utero-ovarian venous plexus enters the hilum of the ovary through the
287 ovarian pedicle (Fig. 2A). Microscopic examination of the vascular pedicle showed
288 sympathetic nerves running with the ovarian vessels (Fig. 2B). Sometimes there was one
289 discrete nerve in the ovarian pedicle (Fig. 2C) but commonly there were several smaller

290 nerves (Fig. 2D-F) from 50 μm to 350 μm in diameter. There were no other neurovascular
291 entry points to the ovary outside the hilum and ovarian pedicle. In each case at least one
292 nerve ≥50 μm could be identified consistently with the ovarian artery. Sympathetic
293 innervation of the ovine ovary runs along the vascular bundle in the ovarian pedicle.
294
295 *The location of sympathetic nerve fibres within the ovary*
296
297 The sympathetic nerves enter the ovary at the hilum as discrete nerves next to blood vessels
298 (Fig. 3A) and can be seen as discrete nerve fibre bundles within the ovarian medulla (Fig.
299 3B). Sympathetic nerves branch further within the ovary and are associated with arterioles
300 and small blood vessels (Fig. 3C-E). Nerve fibres are also seen in the cortical regions of the
301 ovary, in the vicinity of primordial and primary follicles, that are not associated with blood
302 vessels (Fig. 3F). Overall, 40% of nerves identified within the ovary were not associated with
303 blood vessels. There is sympathetic innervation that is independent from blood vessels
304 around primordial, primary and secondary follicles in the ovarian cortex.
305
306 *Regeneration of ovarian nerves after denervation*
307
308 If sympathetic nerves have a physiological role in ovarian function it would be expected that
309 they would regenerate after ovarian denervation. We examined sympathetic nerves in the
310 ovine ovary after oophorectomy, whole ovary cryopreservation and ovarian transplantation
311 (18). The ovaries were disconnected from the neurovascular bundle and thus denervated.
312 After transplantation back onto the ovarian pedicle the ovaries became functional (18). Ten
313 months after transplant histological analysis of ovaries (n=4) showed that all ovaries had

314 discrete nerves (50 µm) at the hilum (Fig. 4A) and a normal distribution of nerves throughout
315 the ovarian stroma, including association with arterioles (Fig. 4B) and cortical nerves
316 independent of blood vessels (Fig. 4B). After denervation the ovine ovary is reinnervated in
317 situ.
318
319 *Acute denervation of the ovine ovary*
320
321 In order to determine if we could acutely denervate the ovine ovary, ewes (n=3) underwent
322 laparotomy and unilateral denervation in the mid-follicular phase using monopolar needle
323 micro-diathermy of the putative ovarian nerves within the neurovascular bundle leaving the
324 vasculature intact. After 21 days the sheep were killed and the ovaries examined. There was
325 a loss of sympathetic nerve fibre immunostaining within the treated ovary compared to the
326 contralateral control ovary (Fig. 5A-C). In addition, there was reduction in intraovarian
327 noradrenaline concentrations (Fig. 5D) that correlated with tissue immunostaining score
328 ($r=0.8407$; $P<0.05$; Fig. 5E). Needle diathermy of the sympathetic nerves in the ovarian pedicle
329 can be used to acutely denervate the ovary.
330
331 *The acute effects of ovarian denervation*
332
333 A separate cohort of ewes were randomised to either bilateral denervation using micro-
334 diathermy (n=4) or a sham procedure without diathermy (n=4). The hormonal profiles of the
335 ewes were then examined daily over an ovarian cycle and ovaries were collected 21 days later
336 and at that stage we examined intraovarian noradrenaline concentrations to confirm ongoing
337 denervation during the experiment. In the follicular phase there was no difference in estradiol
338 concentrations (Fig. 6A). There was no effect of denervation in the timing of ovulation, post-

339 ovulatory progesterone concentrations and luteolysis (Fig. 6B,C). Analysis of the tissue
340 immunostaining score for sympathetic nerves (P<0.01; Fig. 6E) and intraovarian
341 noradrenaline concentrations (P<0.005; Fig. 6F) confirmed ovarian denervation after micro-
342 diathermy.

343 Follicles were counted in a representative mid-ovarian section from each ovary. There
344 were no differences in antral follicle numbers (Fig. 6G) although there was a strong trend to
345 less preantral follicles after diathermy but this didn't reach statistical significance (P=0.056;
346 Fig. 6H). There were no differences in proportion of atretic antral follicles, assessed by cleaved
347 caspase 3 expression (Fig. 6I,J) or growing antral follicles, assessed by Ki67 localisation (Fig.
348 6K,L). In addition, there were no differences in the ovarian transcript abundance of *CCN2*,
349 *VEGFA*, *NGF*, *IGF1* and *FGF2* (Fig. 6M). Acute denervation does not have any impact on
350 follicular growth, ovulation and luteolysis or on the survival of antral follicles.

351

352 *Development of an acute ovine model of PCOS*

353

354 To develop an acute ovine model of PCOS, in normal cycling sheep, we first synchronised the
355 sheep with progesterone sponges then switched off the hypothalamic pituitary ovarian axis
356 using a GnRH agonist (n=3). Sheep were given an infusion of FSH with baseline endogenous
357 LH (Control) or with additional high dose pulsatile LH (PCOS-like). After seven days the ovaries
358 were collected and analysed by optical tomography, which allows the whole ovary to be
359 viewed in real-time digital sections to accurately count and measure all the antral follicles
360 (Fig. 7A,C). These follicles could be identified in subsequent tissue sections, and had a normal
361 follicular structure including healthy granulosa cell and theca cell layers (Fig. 7A,B), after
362 further fixation and sectioning after OPT was complete. There was a different pattern of

363 follicles in the PCO-like ovaries (P<0.0001) with an increased number of smaller and a reduced
364 number of larger follicles, suggesting a polycystic morphology (Fig. 7C,D,F). Gonadotrophin
365 manipulation can facilitate the acute development of polycystic ovaries.
366
367 *The effect of denervation in the acute PCOS model*
368 After bilateral denervation we then assessed the effects of gonadotrophin infusion to create
369 the acute PCOS-like model. There was a difference in the pattern of follicles in the control
370 high dose pulsatile LH PCOS-like sheep (Fig. 8A) compared to the control low LH sheep (Fig.
371 8B). However, denervation showed no difference in the pattern or number of follicles in the
372 high LH PCOS-like sheep (Fig. 8C) or the control low LH sheep (Fig. 8D). Denervation had no
373 effect on gonadotrophin action in the development of a PCO ovary.
374
375
376 **Discussion**
377
378 We have shown that there is dense sympathetic innervation in the ovine ovary that is not
379 only associated with blood vessels but also seen around the avascular small follicles.
380 Sympathetic denervation, confirmed by intra-ovarian sympathetic nerve immunostaining
381 and NA measurement, has no effect on antral follicle growth, ovulation or luteolysis. In
382 addition, it had no effect on gonadotrophin action in follicular development. We recreated a
383 polycystic ovarian morphology using gonadotrophin manipulation to test the effect of
384 sympathetic denervation. There was no effect on the acute antral follicle response. This has
385 narrowed down the potential roles of the sympathetic nervous system in the ovary.
386 Sympathetic innervation of the ovary is seen in multiple species including rodents (26),
387 ruminants (27), non-human primates (28) and women (29). In polyovulatory species such as

388 rodents and pigs there is very clear innervation with easy identification of the superior
389 ovarian nerve (23,30). We hypothesised that this may suggest that sympathetic innervation
390 may protect follicles from atresia, or promote early follicular development, and this may
391 have a role in the development of a polyfollicular (polycystic) ovary. We used a clinically
392 realistic ovine model as the sheep has a robust track record in clinically relevant ovarian
393 research (19, 21, 31).
394 We surmised that total severance of the sympathetic nerves entering the ovary would
395 occur during oophorectomy (19). If ovarian innervation was important for normal ovarian
396 function then nerves would regrow into the ovary after auto-transplantation. The presence
397 of a normal intraovarian sympathetic nerve distribution after resumption of ovarian activity
398 post transplantation does suggest a relevant role for ovarian sympathetic innervation. It has
399 been postulated that ovarian innervation is involved in regulating local gonadotrophin
400 action, either directly (7,8) or though regulation of vascular blood flow. Indeed, there is
401 some evidence that cells within ovarian follicles have some features of nerve cells (32) and
402 cells within the follicle express receptors to NA (33). In rodents, stimulation of the
403 sympathetic nerves increased the number of antral follicles (9). This supports a role for the
404 sympathetic nervous system in supporting follicular growth. Unfortunately, our assay for
405 testosterone was not sensitive enough to allow us to examine the effect of denervation on
406 androgen secretion. It has been suggested that sympathetic nerves facilitate LH-dependent
407 androgen secretion (8), and it is the androgens that are important in the development of a
408 polycystic ovary (34). We cannot say if denervation reduced androgens but showed there
409 was no effect on estrogen levels and importantly no effect on the development of a
410 polycystic ovary induced by increased LH concentrations.

411 We were able to denervate the ovary and examine what happened during the
412 follicular and luteal phase of a cycle. Follicular growth and ovulation occurred normally.
413 Importantly luteolysis also occurred normally. As luteolysis involves a vascular counter-
414 current between the uterus and the ovary (35) this would suggest there was no acute effect
415 on the vasculature that might have delayed luteolysis (19). This suggests that in the absence
416 of sympathetic stimulation gonadotrophins can independently drive mid-late follicular
417 growth and ovulation.

418 If the sympathetic nervous system can augment gonadotrophin action in the ovary,
419 and promote follicular survival, this might facilitate the development of a polycystic ovary in
420 PCOS. Women with PCOS have increased LH action within the ovary (2). Manipulation of LH
421 concentrations can impact on follicular growth and development (36). We hypothesised that
422 increasing LH action in the ovary would increase the number of follicles but block the
423 growth of large antral follicles, developing a polycystic ovary. Using OPT allows every antral
424 follicle within the whole ovary to be measured and counted. Driving the ovary with
425 increased LH concentrations resulted in a different pattern of follicular growth and the
426 development of a macroscopic polycystic ovary.

427 This acute model of PCO allowed us to determine the effects of sympathetic
428 denervation on LH action in the development of a polycystic ovary. We hypothesised that
429 after denervation the polycystic ovary would have less and larger follicles. Elegant rodent
430 studies involving reducing sympathetic innervation improved ovarian function in an induced
431 polycystic ovary phenotype (29). There was no acute effect on ovarian morphology and the
432 denervated ovary developed the same PCO morphology is response to gonadotrophin
433 manipulation as the innervated ovary. Overall this suggests that sympathetic nerves in a
434 large animal, human-like, ovary are not involved in the gonadotrophin dependent phase of

435 follicular growth (4). It remains possible that these nerves do have a function as growth
436 and/or survival factors for smaller gonadotrophin independent follicles. Denervation
437 showed a trend towards a reduction in preantral follicles in the short term.
438 In summary we have developed an acute model of PCO ovarian morphology in an
439 ovine model by manipulating gonadotrophins, which may have future utility in terms of
440 separation of metabolic aspects from ovarian aspects of this syndrome. One benefit of this
441 model was that it allowed us to investigate the role of the sympathetic nervous system in
442 the regulating gonadotrophin action in the follicle. Gonadotrophins are the master regulator
443 of the gonadotrophin dependent follicle and there does not seem to be any significant
444 neural contribution. However, it remains likely that sympathetic action is involved in ovarian
445 function in concert with gonadotrophins. The involvement of sympathetic nerves in the
446 polycystic ovary, and a physiological effect of testosterone secretion, remains possible but
447 that gonadotrophin action is the fundamental driver of ovarian structure and function.
448 There may be effects before gonadotrophins take over follicular growth and development
449 and possible effects on local androgen production that are not able to be ascertained in this
450 model. This suggests that longer term experiments, perhaps using a clinically realistic
451 prenatally programmed ovine model of PCOS (20, 24) would need to be used to dissect the
452 role of the sympathetic nervous system on the survival of smaller follicles, follicular
453 steroidogenesis and the development of the polycystic ovary.
454
455
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461

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579 **Figure Legends**

580

581 **Figure 1**

582 **Fig. 1** Illustration of the protocols used in the development and manipulation of a polycystic

583 ovary. **A**) Validation of the model. **B**) Using the model to test the effect of ovarian

584 denervation.

585

586 **Figure 2**

587 **Fig. 2** Sympathetic nerve supply to the ovine ovary. **A)** Photograph of an ovine ovary (white

588 arrow) in situ highlighting the neurovascular pedicle (red arrow). **B)** Transverse section

589 through the ovarian pedicle stained with tyrosine hydroxylase (brown) showing several

590 discrete sympathetic nerves within the pedicle. **C)** A large sympathetic nerve (brown) in the

591 neurovascular pedicle. **D-F)** Smaller sympathetic nerves (brown) within the pedicle. Scale

592 bar B-D = 50 µm, E,F = 20 µm.

593

594 **Figure 3**

595 **Fig. 3** Sympathetic nerve supply within the ovine ovary. **A)** Confocal staining of the hilar

596 region of the ovary stained for tyrosine hydroxylase (sympathetic nerves) in green and CD-

597 31 (endothelial cells) in red. An arteriole is highlighted by the white arrow. **B)** A discrete

598 sympathetic nerve (brown) within the ovarian stroma. **C)** Nerves (brown) seen around blood

599 vessels and a small preantral follicle (arrows). **D)** Small arterioles with endothelial staining

600 (red) with clear sympathetic nerves (green) surrounding in transverse view. **E)** The plexus of

601 sympathetic nerves (brown) around an arteriole in longitudinal view. **F)** The presence of

602 sympathetic nerves throughout the ovarian cortex and around small preantral follicles. Scale

603 bar = 50 µm.

604

605 **Figure 4**

606 **Fig. 4** Sympathetic nerve supply to the ovary after transplantation. **A)** Larger nerves (arrow)

607 stained for tyrosine hydroxylase (brown) in the hilar region of the ovary post

608 transplantation. **B)** Plexus of sympathetic nerves (brown) around blood vessels in the

609 medulla (arrow). **C)** Sympathetic nerves in the cortex close to primordial follicles (arrow).

610 Scale bar = 50 µm.

611

612 **Figure 5**

613 **Fig. 5** Denervation of the ovary using needlepoint diathermy. **A)** Ovarian stroma stained for

614 tyrosine hydroxylase (brown) highlighting sympathetic nerves that was histoscored blindly

615 as 3. **B)** Contralateral ovary stained for tyrosine hydroxylase after diathermy for denervation

616 showing no specific immunostaining, with a histoscore of 0. **C)** Blinded tissue score for

617 immunostaining for tyrosine hydroxylase in control (C) ovary and diathermy (D) ovary. **D)**

618 Tissue noradrenaline concentrations in control (C) ovary and diathermy (D) ovary. **E)**

619 Significant correlation between noradrenaline concentrations and tissue immunostaining

620 score for noradrenaline. Scale bar = 50 µm.

621

622 **Figure 6**

623 **Fig. 6** The effect of denervation on ovarian structure and function. **A)** Peak estradiol before

624 ovulation in control (C, n=4) and after ovarian denervation (D; n=4). **B)** progesterone

625 dynamics across the luteal phase after ovulation in control (C) and ovarian denervation (D)

626 sheep. **C)** Total progesterone secretion across the luteal phase in control (C, n=4) and after
627 ovarian denervation (D; n=4). **E)** Significant reduction in tissue immunostaining score and **F)**
628 ovarian noradrenaline concentrations after denervation (each ovary is analysed separately).
629 **G)** No significant difference in number of antral follicles or **H)** preantral follicles in
630 representative mid ovarian tissue section. **I)** Representative immunostaining for cleaved
631 caspase-3 (brown) identifing follicular atresia. **J)** Quantification of antral follicles positive for
632 cleaved caspase-3 in control ovaries (C) and after ovarian denervation (D). **K)** Representative
633 immunostaining for Ki67 (brown) identifying growing follicles. **L)** Quantification of antral
634 follicles positive for Ki67 in control ovaries (C) and after ovarian denervation (D). **M)**
635 Transcript abundance in ovarian stroma for key ovarian growth factors in control ovaries (C)
636 and after ovarian denervation (D). ** $P<0.01$, *** $P<0.005$, n.s = not significant, scale bar =
637 50 µm.
638

639 **Figure 7**

640 **Fig. 7** Acute modelling of PCOS. **A)** Static image of optimal tomography whole ovarian scan
641 with **B)** the same area of the ovary after tissue sectioning and haematoxylin and eosin
642 staining after FSH infusion with low LH. **C)** Static image of optimal tomography whole
643 ovarian scan with **D)** the same area of the ovary after tissue sectioning and haematoxylin
644 and eosin staining after FSH infusion with high pulsatile LH. **E)** Cumulative follicles in the
645 whole ovary based of size after FSH infusion with low LH (n=3). **F)** Cumulative follicles in the
646 whole ovary based of size after FSH infusion with high pulsatile LH (n=3).
647

648 **Figure 8**

649 **Fig. 8** The effect of ovarian denervation on acute model of PCOS. A) Average number of

650 antral follicles per ovary after FSH infusion with high pulsatile LH (n=3), B) or after FSH

651 infusion with low LH (n=3), after sham procedure. C) Average number of antral follicles per

652 ovary after FSH infusion with high pulsatile LH (n=3), D) or after FSH infusion with low LH

653 (n=3), after bilateral ovarian denervation procedure.

654