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1 **The effects of small litter rearing on ovarian function at puberty and adulthood**
2 **in the rat**

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12 **Short title:** Ovarian function in small litter reared rats

13

14 **Abstract**

15 Rearing rats in small litters leads to obesity and reproductive dysfunction. We
16 investigated the effects of rearing female rats in small litters on various reproductive
17 parameters during puberty and into adulthood, and examined the possible
18 involvement of local ovarian sympathetic nerve activity. The litter size was adjusted
19 on postnatal day one to four pups per dam for the small litters and 12 pups per dam
20 for the normal litters. Vaginal opening was recorded, and estrous cyclicity was
21 monitored daily immediately post puberty for 14 days and again at 8-9 weeks of age.
22 At the time of puberty and 10 weeks of age, the ovaries were collected. The number
23 of different types of follicles was counted and the thickness of the theca interna of the
24 largest antral follicles was measured. Ovarian sympathetic nerve activity was assessed
25 immunohistochemically by measuring levels of ovarian nerve growth factor receptor
26 (p75NGFR) and tyrosine hydroxylase (TH). In rats reared in small litters, there was a
27 significant advancement of puberty and disruption of estrous cyclicity immediately
28 post puberty. The number of antral follicles increased in the small litter reared rats at
29 the time of puberty compared with their controls. The thickness of the theca interna
30 increased and the expression profiles of ovarian p75NGFR and TH increased in small
31 litter reared rats at puberty, but this did not persist into adulthood. These data suggest
32 that rearing rats in small litters leads to irregular reproductive cycles, which might
33 involve increased local ovarian sympathetic nerve activity.

34 **Key Words:** ovary, obesity, puberty, follicle, sympathetic.

35

36 **1. Introduction**

37 Postnatal overfeeding induced by rearing animals in small litters results in a dramatic
38 increase in body weight gain and programs for overweightness and persistent
39 hyperphagia in adulthood, even though a standard diet was provided after weaning [1].
40 It is well established that reproductive function is gated by the state of energy reserves
41 of the organism. The timing of puberty in mammals is tightly coupled to the animals'
42 nutritional and metabolic state. Food restriction in female rats delays puberty [2,3],
43 while female rats reared in small litters have been shown to display early puberty
44 onset [1,4]. Obesity also affects female reproductive function by affecting
45 spontaneous ovulation in humans and animals. The relationship between excess body
46 fat and reproductive disorders in women appears to be stronger for early-onset obesity
47 during their life, particularly during adolescence [5]. There is evidence that in
48 adolescent and young women, the age of onset of obesity and of menstrual
49 irregularities and oligo-anovulation are significantly correlated [5,6]. The prevalence
50 of obesity in women with PCOS appears to be much greater than expected in the
51 general population. Studies in the cafeteria diet-overfed [7] and genetically obese
52 Zucker female rats [8] have shown that these animals display a disruption of estrous
53 cyclicity as well as obesity in adulthood. However, the mechanism of how obesity or
54 overweight link to ovarian dysfunction is not established and there is a sparse
55 literature on the effect of rearing in small litters on ovarian activity in the rat.
56 Rats reared in small litters has been shown to accumulate more noradrenaline in the
57 heart than animals reared in larger litters [9], with cardiac noradrenaline

58 concentrations inversely related to litter size at 40 days of age [10]. It was shown that
59 noradrenaline levels were also increased in hypothalamus [11] and ovary in the rat [12]
60 during puberty. Abnormally increased noradrenaline levels [13] and tyrosine
61 hydroxylase (TH) immuno-staining in ovary has been demonstrated to cause ovarian
62 dysfunction such as early vaginal opening, disrupted estrous cyclicity and appearance
63 of cystic follicle in the polycystic ovary (PCO) rat [14]. Transection of the superior
64 ovarian nerve decreases noradrenergic innervation of the ovary and restores estrous
65 cyclicity and ovulation in this model [15]. Moreover, increased density of
66 catecholamine nerves has been observed in the ovaries of PCO patients [16] and the
67 marked effectiveness of ovarian wedge resection to initiate ovulatory cycles in PCO
68 patients further supports the importance of sympathetic activity on ovarian function
69 [17,18]. However, there is no literature on whether the ovarian sympathetic nerve
70 activity was affected in rats reared in small litters.

71 The aim of this study is to test the hypothesis that rearing female rats in small litters
72 as a model of postnatal overnutrition, which advances puberty, is associated with
73 ovarian dysfunction at puberty and in adulthood, as manifested by alterations in
74 ovarian cyclicity and morphology and expression of ovarian nerve growth factor
75 receptor (p75NGFR) and TH; markers of sympathetic nerve activity.

76 **2. Materials and Methods**

77 **2.1 Animal procedure**

78 Pregnant Sprague-Dawley rats (Charles River, Manston, UK) were housed under
79 controlled conditions (12 h of light and 12 h of darkness with lights on at 07:00 h and

80 a controlled ambient temperature of $22 \pm 2^{\circ}\text{C}$) and supplied with *ad libitum* food and
81 water. On postnatal day (pnd) 1 (birth, pnd 0) litter size was adjusted to 4 pups per
82 dam for the small litter reared group ($n = 7$), and to 12 pups per dam for the normal
83 litter reared group ($n = 6$). At least 1 male pup was included in each litter. The dams
84 were singly housed during pregnancy and lactation, with weaning on pnd 21. Post
85 weaning, 4-6 pups were housed in the same cage until they reached 10 weeks of age.
86 They had free access to water and food. All animal procedures were conducted under
87 the British Home Office Animal Scientific Procedure Act 1986 (Project Licence 0671)
88 and in accordance with accepted standards of the local ethical review committee.

89 **2.2 Puberty onset and estrous cyclicity monitoring**

90 Animals were monitored daily for vaginal opening from pnd 28. Once vaginal
91 opening occurred, vaginal smears were taken and monitored daily for 2 consecutive
92 weeks and again at 8-9 weeks of age. The criteria for normal estrous cyclicity were
93 the same as we described previously [19]. Animals were weighed weekly until the end
94 of the experiment.

95 **2.3 Ovarian morphology**

96 Pubertal ovaries were collected on pnd 42 and adult ovaries were collected at diestrus
97 at the age of 10 weeks. The right ovary was cleaned of fat tissue, weighed and fixed in
98 10% formaldehyde buffer for 20 h at room temperature. Wax-embedded ovaries were
99 sectioned longitudinally at $4\mu\text{m}$ and mounted on glass slides. For counting the number
100 of the different types of follicles, every other section was stained with
101 haematoxylin-eosin and subjected to analysis. Only follicles in which the nucleus of

102 the oocyte was visible were counted [20]. The thickness of the theca interna layer of
103 the largest follicle was determined in every tenth section (six sections per ovary) with
104 a calibrated scale bar in the microscope. The sections were examined under a light
105 microscope (Zeiss Axioskop 2 plus, Oberkochen, Germany) with an image analysis
106 system (Axiovision 2.05; Zeiss) by two independent investigators blind to the
107 treatment group. The follicles were classified as following: primordial -follicles with
108 oocytes surrounded by one layer of flattened pregranulosa cells; primary-follicles with
109 oocytes surrounded by no more than two layers of cuboidal granulosa cells; preantral
110 follicles without any antral cavity and with two or more layers of granulosa cells;
111 antral-follicles with apparent cavity [20].

112 **2.4 Immunohistochemistry**

113 Immunohistochemical staining for p75NGFR and TH were performed on 4µm
114 paraffin embedded sections from ovaries of 6- and 10-week-old rats. The sections
115 were deparaffinised in xylene, hydrated in descending concentration of ethanol and
116 incubated with 0.3% Triton X-100 (Sigma-Aldrich) in PBS to increase permeability
117 before incubated with 0.3% H₂O₂ to inhibit endogenous peroxidases and with
118 non-immune goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS to
119 reduce background staining. The sections were then incubated in 1:1000 monoclonal
120 mouse anti-p75NGFR primary antibody (Chemicon International, Temecula, CA,
121 USA) containing 2% normal goat serum at 4° C for 24 h. (or 1:1500 polyclonal rabbit
122 anti-TH primary antibody containing 2% normal goat serum at 4°C for 36 h). The
123 sections were then rinsed in 1:150 biotinylated goat anti-mouse IgG (Vector

124 Laboratories) for 90 min (or 1:300 goat anti-mouse IgG for 120 min) at room
125 temperature followed by 1:200 conjugated avidin-biotin complex (Vector Laboratories)
126 for additional 45 min at room temperature. Visualisation of p75NGFR and TH
127 immunoreactivity was achieved using the 3',3'-diaminobenzidine (Sigma-Aldrich).
128 The sections were counterstained with 10% haematoxylin and coverslipped with DPX.
129 Negative controls were run by incubating adjacent sections with non-immune serum
130 and omission the primary antibody. The sections from the normal litters and small
131 litters were run together in the same batch. Immunohistochemical detection of rat
132 spinal cord and adrenal were included as a positive control for p75NGFR and TH in
133 each batch of experiments. In each immunohistochemical experiment, two control
134 sections from the same ovary tissue were included as immunostaining quality controls
135 for the variation between different batches of experiments.

136 Six sections per ovary from each rat were used for immunostainings and five fields of
137 vision from each section were analyzed under optical microscope. Semi-quantitative
138 analyses of immunostaining intensity for p75NGFR and TH were carried out on a
139 Zeiss AxioVision microscope image system (Zeiss). All analyses were performed on
140 coded slides by two independent investigators blinded to the treatment groups. The
141 intensity of immunostaining of p75NGFR and TH were assessed by using a
142 modification of a semi-quantitative *H*-Score method described by Akercan *et al* [21].
143 Semi-quantitative immunohistochemical *H*-score values were calculated from the
144 intensity and percentage of cells staining at each intensity. Intensities were classified
145 as 0 (no staining), +1 (weak staining), +2 (weak-moderate staining), +3 (moderate

146 staining), +4 (strong staining), +5 (very strong staining). For each slide, the *H*-Score
147 was calculated using the equation: $\sum(I \times PC)$, where *I* and *PC* represent intensity and
148 percentage of cells that stain at each intensity respectively. Data were expressed as
149 mean \pm S.E.M. The different groups were analyzed by the Mann-Whitney *U* test. *P*
150 values <0.05 were considered statistically significant.

151 **2.5 Statistical analysis**

152 Comparisons between the small litter and normal litter reared groups in terms of body
153 weight, vaginal opening and first vaginal estrus were made by subjecting data to
154 one-way ANOVA followed by Dunnett's test. The percentage of normal estrous cycles
155 between groups was compared using X^2 test. Comparisons between groups on
156 thickness of the theca interna layer were made by subjecting data to the
157 Mann-Whitney *U* test. All data are shown as mean \pm S.E.M. *P* values <0.05 were
158 considered statistically significant.

159 **3. Results**

160 **3.1 Rearing animals in small litters advanced vaginal opening and first vaginal** 161 **estrus**

162 The gain in body weight was significantly greater in small litter compared with
163 normal litter reared rats throughout development, although there was no difference at
164 week one postpartum. The significant difference in body weight persisted into
165 adulthood (Fig.1).

166 For the timing of puberty, rearing female rats in small litters significantly advanced

167 both the day of vaginal opening (small litter: 34.3 ± 0.3 , n=20; normal litter: $36.3 \pm$
168 0.3 , n=22; $P < 0.05$) and the day of first vaginal estrus (small litter: 34.8 ± 0.5 , n=20;
169 normal litter: 36.5 ± 0.3 , n=22; $P < 0.05$).

170 **3.2 Rearing animals in small litters disrupted estrous cyclicity at puberty without** 171 **persisting into adulthood**

172 Representative examples of estrous cycles in each group are illustrated in Fig. 2A and
173 B. The majority (66.4%) of rats reared in the normal litter group showed a typical 4-5
174 day estrous cycles at puberty, and this percentage increased to 77.8% in adulthood
175 (Fig. 2C). In rats reared in small litters, only 35.0% displayed normal cyclicity at
176 puberty, which improved to 65.0% in adulthood. The percentage of normal estrous
177 cycles was significantly lower in the small litter reared group at puberty (Fig. 2C).

178 **3.3 Effect of rearing animals in small litters on ovarian weight and morphology** 179 **at puberty and in adulthood**

180 The mean ovarian weight at puberty (pnd 42) for the normal litter and small litter
181 reared groups was 36.80 ± 3.81 mg (n=20) and 35.53 ± 3.02 mg (mean \pm S.E.M, n=22)
182 respectively. At adulthood (10 weeks), ovarian weight for the normal litter and small
183 litter reared animals was 71.41 ± 8.32 mg (n=20) and 72.23 ± 10.24 mg (mean \pm S.E.M,
184 n=22), respectively. There was no significant difference in ovarian weight between the
185 small and normal litter reared rats either at puberty or in adulthood. There was no
186 significant difference in the mean number of primordial, primary or preantral follicles,
187 or corpora lutea either at puberty or adulthood (Table 1). However, the number of
188 antral follicles was increased at puberty in the small litter reared rats compared with

189 the normal litter reared animals (Table 1).

190 The thickness of the theca interna layer of the largest follicle was significantly
191 increased in ovaries from the small litter reared group at puberty (Fig. 3A, B, C), but
192 this difference did not persist into adulthood (Fig. 3D).

193 **3.4 Effect of rearing animals in small litters on sympathetic tone in the ovary of** 194 **pubertal and adult rats.**

195 The immunohistochemical staining for p75NGFR was generally confined to the theca
196 cells, especially theca interna cells of growing follicles (preantral and antral follicles;
197 Fig. 4A-D). Neither primordial nor primary follicles had specific staining for
198 p75NGFR. There was no difference in the staining pattern between ovaries from the
199 small litter reared and normal litter reared groups. However, the intensity of staining
200 for p75NGFR in preantral and antral follicles was enhanced at puberty in the small
201 litter compared with the normal litter reared control animals (Fig. 4E). In adulthood
202 (diestrus) this difference was no longer evident.

203 The immunostaining for TH was distributed in theca cells of preantral and antral
204 follicles, corpora lutea, and interstitial tissue. The staining pattern for TH in ovaries
205 from rats reared in small litters was the same as that for normal litter controls.
206 However, the staining intensity for TH (theca cells) in preantral follicles was
207 enhanced in ovaries from the small litter reared group at puberty (Fig. 5A-E),
208 compared with the normal litter controls (Fig. 5E), whereas in adulthood (diestrus)
209 this difference was no longer evident.

210 **4. Discussion**

211 This study demonstrates that rearing female rats in small litters can have effects on
212 ovarian morphology and reproductive function at puberty, without persisting into
213 adulthood. Not only were markers of puberty onset (vaginal opening and first vaginal
214 estrus) advanced, but in addition, estrous cyclicity was disrupted at puberty. The
215 disruption to estrous cyclicity was characterized by a persistent or prolongation of
216 vaginal estrus. A remarkable increase in the number of antral follicles was observed in
217 the ovaries of the small litter reared rats compared with their controls at puberty.
218 However, at the adult stage this difference was no longer evident. The thickness of the
219 theca interna layer and the markers of sympathetic tone, including p75NGFR and TH
220 immunoreactivity, in the ovary were increased at the time of puberty in rats from the
221 small litter reared group, but were not evident in adulthood. In our study, postnatal
222 overfeeding by rearing rats in small litters resulted in obesity throughout life, which is
223 in accordance with other studies [22]. Puberty onset is closely correlated with
224 preweaning nutrition and body weight gain [23]. As expected, there is a strong
225 correlation between body weight and age at vaginal opening as previously observed
226 [1,4]. Overnutrition due to small litter rearing as in the present study, or high fat diet
227 feeding, induces a persistent increase in body weight gain and advances puberty [1,4,
228 24]. Conversely, negative energy balance due to excess exercise or food restriction
229 delays puberty [4,25]. Although the underlying mechanism controlling the timing of
230 puberty onset which is influenced by metabolic cues and nutritional status remains
231 undefined, it appears that appropriate body weight and adiposity are necessary for

232 puberty to occur [23,26]. Leptin and kisspeptin are affected by adiposity levels and
233 unequivocally required for the onset of puberty. Postnatal overnutrition by rearing rats
234 in small litters is associated with higher levels of leptin and increased levels of
235 hypothalamic kisspeptin mRNA expression at puberty [1], although others report no
236 affect of small litter rearing on hypothalamic levels of kisspeptin expression at
237 puberty [4]. Complex changes in the regulatory mechanisms of the hypothalamic-
238 pituitary-gonadal axis with overnutrition, may attribute to the earlier accelerated
239 GnRH pulse generator frequency to advance puberty [24].

240 The finding of abnormal estrous cyclicity at puberty in the small litter reared rats
241 suggests abnormal follicular development and ovarian steroid production. Although
242 the impact of postnatal overnutrition, by rearing in small litters, on ovarian follicular
243 morphology has not previously been studied in the rat, an increase in the number of
244 the small antral follicle in ovaries of young adult rhesus monkeys fed a Western-style
245 diet was reported recently [27]. We found in the current study that the number of
246 antral follicles was markedly increased, by more than 100%, at puberty in the small
247 litter reared rats. However, this difference was lost in the adult rat and may therefore
248 explain why a significantly higher number of irregular estrus cycles were detected
249 only immediately after puberty, but not at the young adult stage in the small litter
250 reared animals. This is consistent with a recent study showing that rearing in small
251 litters did not alter the duration of the estrous cycles in the young adult female rats
252 [28]. The mechanism for the increased number of antral follicles in the ovary of the
253 small litter reared rat could be a direct malfunction of the reproductive system

254 induced by overnutrition at the level of the hypothalamus, pituitary, or ovary either
255 independently or in combination. It is generally accepted that the rise in FSH controls
256 the dynamics of small antral follicle growth and the mid-cycle LH surge stimulates
257 ovulation. It has been reported that postnatal overnutrition by rearing rats in small
258 litters increased FSH levels, whilst reducing circulating estradiol levels [28]. Similarly,
259 pituitary expression of FSH β mRNA was elevated in small litter reared rats [28].
260 However, one of the most striking features of women with PCOS is the presence of
261 numerous antral follicles accompanied with an increased LH pulse frequency [29].
262 Previous studies have shown an increase in basal LH levels in small litter reared rats
263 [1], which may impact on other hormonal signals that may in turn attribute to changes
264 in follicle development, luteal formation or sex steroid production in the ovary. Very
265 recently, increased numbers of small antral follicles owing to presence of more atretic
266 follicles, and alterations in the transcriptome of the small antral follicle were detected
267 in young adult monkeys fed with high fat/fructose diet [27], which are similar to those
268 observed in women with obesity and/or PCOS. Furthermore, decreased ovarian
269 reserve, dysregulation of mitochondrial biogenesis, and increased lipid peroxidation
270 has been reported in mouse offspring exposed to an obesogenic maternal diet (high
271 fat/high sugar) [30].

272 The effects on ovarian morphology and sympathetic activity observed immediately
273 post-puberty in rats reared in small litters is presumably regulated by local
274 intra-ovarian mechanisms and/or changes in the activity of neural inputs to the ovary.
275 A single injection of estradiol valerate to neonatal rats was demonstrated to disrupt

276 estrous cyclicity and induce the development of PCO that was causally related to an
277 increase in ovarian noradrenaline concentration [13]. It is interesting to note that this
278 treatment with estradiol valerate did not increase noradrenaline concentration in the
279 celiac ganglion, the central sympathetic innervation to the ovary [13]. It has been
280 shown that the pre-pubertal increase in follicular noradrenaline regulates the follicular
281 response to gonadotrophins and ovulation [31,32], while denervation of the ovary
282 results in a delay of follicular development and puberty onset [20]. Increased
283 sympathetic innervation of the ovary has been shown to disrupt estrous cyclicity and
284 impair follicular maturation [33,34].

285 The increased ovarian sympathetic tone, indicated by the elevated immunoreactivity
286 for TH and p75NGFR especially in the theca interna, in the small litter reared rats
287 may underlying the disruption of estrous cyclicity in the immediate post-puberty
288 period observed in the present study. An up-regulation of sympathetic tone indicated
289 by augmented production of ovarian nerve growth factor was shown to contribute to
290 the formation of ovarian cysts in a rat PCO model induced by estradiol valerate
291 [34,35]. Furthermore, estradiol valerate not only increases intraovarian synthesis of
292 nerve growth factor but its receptor p75NGFR, which was abundantly expressed in
293 both the thecal compartment and nerve fibers of ovaries [35].

294 Although ovarian sympathetic innervation is important for follicular development,
295 steroid secretion and ovarian function [20,36], abnormal increased sympathetic
296 activity in the ovary has also been shown to precede disrupted estrous cyclicity,
297 reduce ovulation and the appearance of cystic ovaries in stress animal models [37]. In

298 the present study, the number of antral follicles and the thickness of the theca interna
299 layer of the largest follicle were increased in the immediate post-puberty period in the
300 small litter reared animals. This may be due to the increased ovarian sympathetic tone
301 since previous studies using chronic intermittent cold stress, which classically
302 enhances ovarian sympathetic activity, showed increased thickness of the theca layer
303 in the rat ovary [37].

304 Human polycystic ovaries have been found to have an increased sympathetic
305 innervation [38] and ovarian wedge resection, especially when compromising the
306 hilum, the point of nerve entry into the ovary, has been shown to be effective in PCOS
307 patients unresponsive to standard treatment with clomiphene citrate [17]. Furthermore,
308 sympathetic hyperactivity in PCOS patients has been detected by indirect [39] and
309 direct measurements of sympathetic nerve mediated ovarian steroid secretion and
310 intraovarian neurotrophic influences on follicular development could have a function
311 in the development of PCOS [16]. Recently, it was shown that reducing sympathetic
312 outflow by exercise or acupuncture decreased the thickened theca interna layer in the
313 dihydrotestosterone-induced PCO rat model [40]. Therefore, the increased markers of
314 ovarian sympathetic activity observed in the present study may underlie the altered
315 ovarian morphology and disruption of estrous cyclicity in the small litter reared rats at
316 puberty. Once these animals reached adulthood, the abnormal estrous cycles and
317 thickened theca interna layer were no longer evident, which correlates with the
318 disappearance of the increased ovarian sympathetic tone markers at this stage.
319 Similarly, in girls PCOS commonly starts at the time of puberty, but symptoms

320 usually relieve or improved as they reach adulthood. The results from our study may
321 help to understand the mechanism underlying PCOS in adolescence.

322 In conclusion, this study shows that rearing rats in small litters, as a model of
323 overnutrition, leads to ovarian dysfunction at puberty, especially disruption of estrous
324 cyclicity, increased numbers of antral follicles and thickening on the theca interna,
325 which might be associated with increased local ovarian sympathetic tone similar to
326 what is observed in women with polycystic ovary.

327 **Declaration of interest**

328 The authors declare that there is no conflict of interest that could be perceived as
329 prejudicing the impartiality of the research reported.

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333

334 **References**

- 335 [1] Castellano JM, Bentson AH, Sánchez-Garrido MA, Ruiz-Pino F, Romero M,
336 Garcia-Galiano D, et al. Early metabolic programming of puberty onset: impact of
337 change in postnatal feeding and rearing conditions on the timing of puberty and
338 development of the hypothalamic kisspeptin system. *Endocrinology* 2011;152(9):
339 3396-3408.
- 340 [2] Bronson FH. Food-restricted, prepubertal, female rats: rapid recovery of
341 luteinizing hormone pulsing with excess food, and full recovery of pubertal
342 development with gonadotropin-releasing hormone. *Endocrinology* 1986;118(6):
343 2483-2487.
- 344 [3] Wade GN, Schneider JE. Metabolic fuels and reproduction in female mammals.
345 *Neuroscience and Biobehavioral Reviews* 1992;16(2):235-272.
- 346 [4] Smith JT, Spencer SJ. Prewaning over- and underfeeding alters onset of puberty
347 in the rat without affecting kisspeptin. *Biology of Reproduction* 2012;86(5): 145,1-8.
- 348 [5] Pandey S, Bhattacharya S. Impact of obesity on gynecology. *Womens Health*
349 (London England) 2010;6(1):107-117.
- 350 [6] Pelusi C, Pasquali R. Polycystic ovary syndrome in adolescents: pathophysiology
351 and treatment implications. *Treatments in Endocrinology* 2003;2(4):215-230.
- 352 [7] Zhou Q, Chen H, Yang S, Li Y, Wang B, Chen Y,et al. High-fat diet decreases the
353 expression of kiss mRNA and kisspeptin in the ovary, and increases ovulating
354 dysfunction in postpubertal female rats. *Reproductive Biology and Endocrinology*
355 2014;12:127.

356 [8] Marín Bivens CL, Olster DH. Abnormal estrous cyclicity and behavioral
357 hyporesponsiveness to ovarian hormones in genetically obese Zucker female rats.
358 *Endocrinology* 1997;138(1):143-148.

359 [9] Iversen LL, De Champlain J, Glowinski J, Axelrod J. Uptake, storage, and
360 metabolism of norepinephrine in tissues of the developing rats. *Journal of*
361 *Pharmacology and Experimental Therapeutics* 1967;157(3):509-516.

362 [10] Seidler FJ, Bell JM, Slotkin TA. Undernutrition and overnutrition in the neonatal
363 rat: long-term effects on noradrenergic pathways in brain regions. *Pediatric Research*
364 1990;27(2):191-197.

365 [11] Matsumoto AM, Karpas AE, Southworth MB, Dorsa DM, Bremner WJ.
366 Evidence for activation of the central nervous system-pituitary mechanism for
367 gonadotropin secretion at the time of puberty in the male rat. *Endocrinology*
368 1986;119(1):362-369.

369 [12] Morimoto K, Okamura H, Tanaka C. Developmental and periovulatory changes
370 of ovarian norepinephrine in the rat. *American Journal of Obstetrics and Gynecology*
371 1986;143(4):389-392.

372 [13] Sotomayor-Zárate R, Dorfman M, Paredes A, Lara HE. Neonatal exposure to
373 estradiol valerate programs ovarian sympathetic innervation and follicular
374 development in the adult rat. *Biology of Reproduction*. 2008;78(4):673-680.

375 [14] Manni L, Holmang A, Lundeberg T, Aloe L, Stener-Victorian E. Ovarian
376 expression of alpha (1)- and beta (2)-adrenoceptors and p75 neurotrophin receptors in
377 rats with steroid-induced polycystic ovaries. *Autonomic Neuroscience: Basic and*

378 Clinical 2005;118(1-2):79-87

379 [15] Barria A, Leyton V, Ojeda SR, Lara HE. Ovarian steroidal response to
380 gonadotropins and beta-adrenergic stimulation is enhanced in polycystic ovary
381 syndrome: role of sympathetic innervation. *Endocrinology* 1993;133(6):2696-2703.

382 [16] Hashim ZH, Hamdan FB, Al-Salihi AR. Autonomic dysfunction in women with
383 polycystic ovary syndrome. *Iranian Journal of Reproductive Medicine* 2015;13(1):
384 27-34.

385 [17] Szilagyi A, Hole R, Keckstein J, Rossmanith WG. Effects of ovarian surgery on
386 the dopaminergic and opioidergic control of gonadotropin and prolactin secretion in
387 women with polycystic ovarian disease. *Gynecological Endocrinology* 1993;7(3):
388 159-166.

389 [18] Costello MF, Ledger WL. Evidence-based management of infertility in women
390 with polycystic ovary syndrome using surgery or assisted reproductive technology.
391 *Womens Health (London England)* 2012;8(3):291-300.

392 [19] Wu XQ, Li XF, Ye BL, Popat N, Milligan SR, Lightman SL, et al. Neonatal
393 programming by immunological challenge: effects on ovarian function in the adult rat.
394 *Reproduction* 2011;141(2):241-248.

395 [20] Lara HE, McDonald JK, Ojeda SR. Involvement of nerve growth factor in female
396 sexual development. *Endocrinology* 1990;126(1):364-375.

397 [21] Akercan F, Cirpan T, Terek MC, Ozcakil HT, Giray G, Sagol S, et al. The
398 immunohistochemical evaluation of VEGF in placenta biopsies of pregnancies
399 complicated by preeclampsia. *Archives of Gynecology and Obstetrics* 2008;277(2):

400 109-114.

401 [22] Stefanidis A, Spencer SJ. Effects of neonatal overfeeding on juvenile and adult
402 feeding and energy expenditure in the rat. *PLoS One* 2012;7(12):e52130.

403 [23] Ahmed ML, Ong KK, Dunger DB. Childhood obesity and the timing of puberty.
404 *Trends in Endocrinology and Metabolism* 2009;20:237-242.

405 [24] Li XF, Lin YS, Kinsey-Jones JS, O’Byrne KT. High-fat diet increases LH pulse
406 frequency and kisspeptin-neurokinin B expression in puberty-advanced female rats.
407 *Endocrinology* 2012;153(9):4422-4431.

408 [25] Foster DL, Jackson LM, Padmanabhan V. Programming of GnRH feedback
409 controls timing puberty and adult reproductive activity. *Molecular and Cellular*
410 *Endocrinology*. 2006; 254-255:109-119.

411 [26] Vizmanos B, Marti-Henneberg C. Puberty begins with a characteristic
412 subcutaneous body fat mass in each sex. *European Journal of Clinical Nutrition*
413 2000;54(3):203-208.

414 [27] Bishop CV, Xu F, Xu J, Ting AY, Galbreath E, McGee WK, et al. Western-style
415 diet, with and without chronic androgen treatment, alters the number, structure, and
416 function of small antral follicles in ovaries of young adult monkeys. *Fertil Steril*. 2015
417 (in press). Epub 2015 Dec 21.

418 [28] Sánchez-Garrido MA, Ruiz-Pino F, Manfredi-Lozano M, Leon S, Heras V,
419 Castellano JM, et al. Metabolic and Gonadotropic Impact of Sequential Obesogenic
420 Insults in the Female: Influence of the Loss of Ovarian Secretion. *Endocrinology*
421 2015;156(8):2984-2998.

422 [29] Blank SK, Helm KD, McCartney CR, Marshall JC. Polycystic ovary syndrome in
423 adolescence. *Annals of the New York Academy of Sciences*. 2008;1135:76-84.

424 [30] Aiken CE, Tarry-Adkins JL, Penfold NC, Dearden L, Ozanne SE. Decreased
425 ovarian reserve, dysregulation of mitochondrial biogenesis, and increased lipid
426 peroxidation in female mouse offspring exposed to an obesogenic maternal diet.
427 *Faseb Journal* 2015(in press). Epub 2015 Dec 23.

428 [31] Aguado LI, Ojeda SR. Prepubertal ovarian function is finely regulated by direct
429 adrenergic influences. Role of noradrenergic innervation. *Endocrinology* 1984;114(5):
430 1845-53.

431 [32] Morales L, Chávez R, Ayala ME, Domínguez R. Effects of unilateral or bilateral
432 superior ovarian nerve section in prepubertal rats on the ovulatory response to
433 gonadotrophin administration. *Journal of Endocrinology* 1998;158(2):213-9.

434 [33] Dorfman M, Ramirez VD, Stener-Victorin E, Lara HE. Chronic-intermittent cold
435 stress in rats induces selective ovarian insulin resistance. *Biology of Reproduction*.
436 2009;80(2):264-71.

437 [34] Dissen GA, Lara HE, Leyton V, Paredes A, Hill DF, Costa ME, et al. Intraovarian
438 excess of nerve growth factor increases androgen secretion and disrupts estrous
439 cyclicity in the rat. *Endocrinology* 2000; 141(3):1073-1082.

440 [35] Lara HE, Dissen GA, Leyton V, Parades A, Fuenzalida H, Fiedler, et al. An
441 increased intraovarian synthesis of nerve growth factor and its low affinity receptor is
442 a principal component of steroid-induced polycystic ovary in the rat. *Endocrinology*
443 2000;141(3);1059-1072.

444 [36] Ojeda SR, Lara HE, editors. The Menstrual cycle and its disorders: role of the
445 sympathetic nervous system in the regulation of ovarian function. Berlin: Springer-
446 Verlag;1989.

447 [37] Dorfman M, Arancibia S, Fiedler JL, Lara HE. Chronic intermittent cold stress
448 activates ovarian sympathetic nerves and modifies ovarian follicular development in
449 the rat. *Biology of Reproduction* 2003;68(6):2038-2043.

450 [38] Heider U, Pedal I, Spanel-Borowski K. Increase in nerve fibers and loss of mast
451 cells in polycystic and postmenopausal ovaries. *Fertil Steril* 2001;75(6): 1141–1147.

452 [39] Garcia-Rudaz C, Armando I, Levin G, Escobar ME, Barontini M. Peripheral
453 catecholamine alterations in adolescents with polycystic ovary syndrome. *Clinical*
454 *Endocrinology(Oxford)* 1998;49(2):221–228.

455 [40] Mannerås L, Cajander S, Lönn M, Stener-Victorin E. Acupuncture and exercise
456 restore adipose tissue expression of sympathetic markers and improve ovarian
457 morphology in rats with dihydrotestosterone-induced PCOS. *American Journal of*
458 *Physiology, Regulatory Integrative Comparative Physiology* 2009;296(4):R1124-
459 R1131.

460

461 **Figure legends**

462 **Figure 1.** Effects of rearing animals in small litters on body weight. The animals
463 reared in small litters show a significant increase in body weight, which persists into
464 adulthood. * $P < 0.05$ versus animals reared in normal litter ($n = 20-22$ pups per group).

465 **Figure 2.** Rearing rats in small litters disrupted estrous cyclicity immediately
466 post-puberty, with improvement in adulthood. Representative examples of estrous
467 cyclicity are illustrated in (A) and (B). The percentage of normal estrous cycles was
468 decreased at puberty but improved in adulthood in rats reared in small litters
469 compared with normal litter size controls (C). P, proestrus; E, estrus; M, metestrus; D,
470 diestrus. * $P < 0.05$ versus animals reared in normal litters as control ($n = 20-22$ pups per
471 group).

472 **Figure 3** Effects of rearing rats in small litters on the thickness of the theca interna
473 layer of the largest follicle in pubertal and adult ovaries. Representative examples of
474 the theca interna layer of the largest follicle in pubertal ovaries are illustrated in A
475 (small litter) and B (normal litter). The thickness of the theca interna layer of the
476 largest follicles was increased at diestrus in rats reared in small litters at puberty (C),
477 but at adulthood (D). CL, corpus luteum; GC, granulosa cell; TC, theca cell; arrows
478 show the theca interna layer. Photomicrographs illustrate diestrus stage of estrous
479 cycle. Scale bar = 50 μm . * $P < 0.05$ versus animals reared in normal litters as control
480 ($n = 10-11$ pups per group).

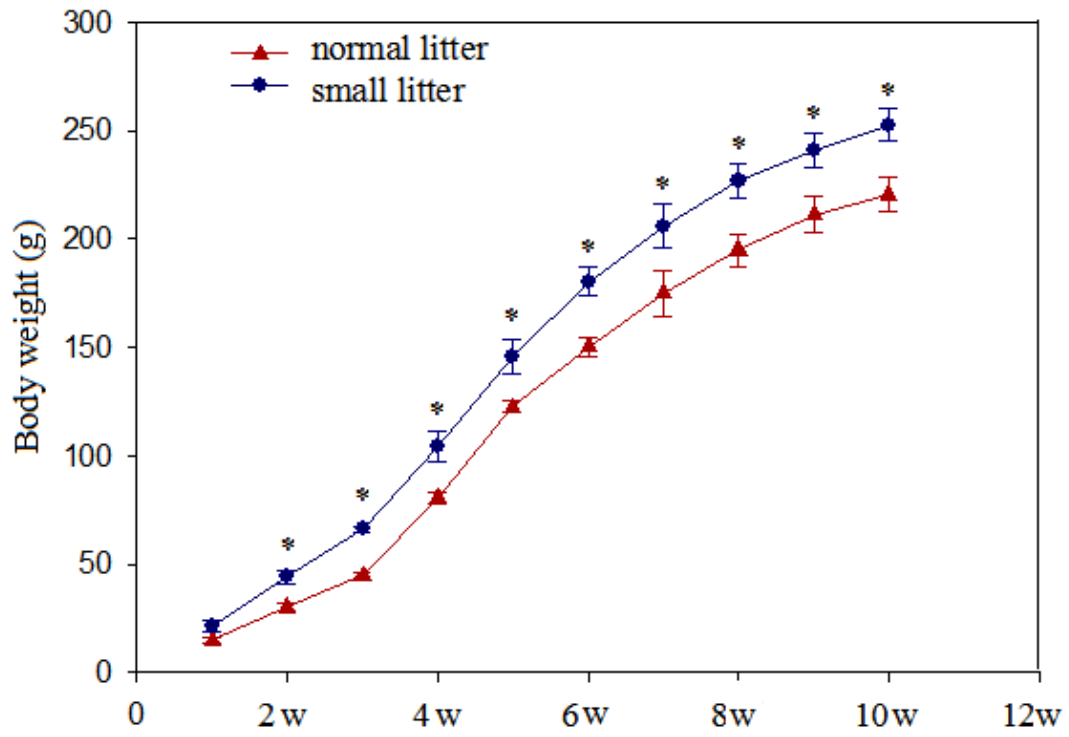
481 **Figure 4** Effects of rearing animals in small litter on the immunoreactivity of
482 p75NGFR in theca interna cells of the pubertal ovary. Representative examples
483 illustrating the expression of p75NGFR protein in theca interna cells of pubertal
484 ovaries from normal litter and small litter reared rats. Theca cells, especially the theca
485 interna (arrows) of growing follicles contain low but detectable levels of p75NGFR
486 protein in normal litter controls (A). The immunostaining for p75NGFR was
487 significantly enhanced in the rats reared from the small litters (B). C, shows a higher
488 magnification of the boxed area in B. D, shows the control section incubated in
489 absence of primary antibody. H-Scores of immunostaining intensity for p75NGFR in
490 theca interna cells are summarised in E. GC, granulosa cell; TCI, theca interna cell;
491 TCE, theca externa cell. Photomicrographs illustrate diestrus stage of estrous cycle.
492 Scale bars: A, B, D=50 μ m; C=25 μ m. * P <0.05 versus animals reared in normal litters
493 as control (n =10-11 pups per group).

494 **Figure 5** Effects of rearing animals in small litter on the immunoreactivity of TH in
495 theca interna cells of the pubertal ovary. Representative examples illustrating the
496 expression of TH protein in theca cells of pubertal ovaries from both small litter and
497 normal litter reared control rats. Theca cells (arrows) of the growing follicles contain
498 low but detectable levels of TH protein in normal litter rats (A). The immunostaining
499 for TH was significantly enhanced in the small litter reared rats (B). C, shows a higher
500 magnification of the boxed area in A. D, shows the control section incubated in
501 absence of primary antibody. H-Scores of immuno-staining intensity for p75NGFR in

502 theca interna cells are summarised in E. The staining intensity was significantly
503 enhanced in the preantral follicles from small litter reared rats compared with normal
504 litter size controls. GC, granulosa cell; TC, theca cell; CL, corpus luteum. Scale bars:
505 A, B, D=50 μm ; C=25 μm . * $P < 0.05$ versus normal litter reared animals as control
506 ($n=10-11$ pups per group).

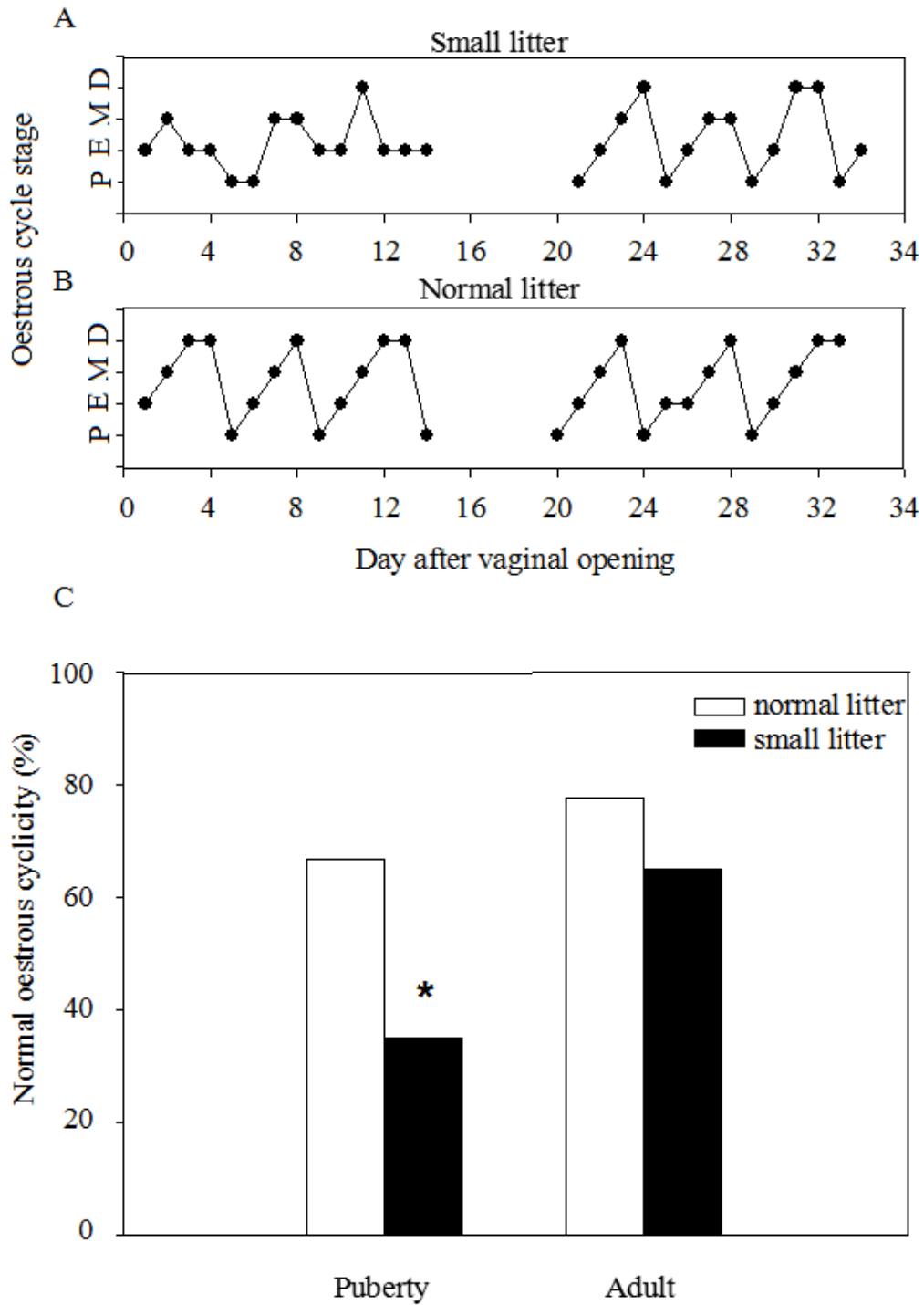
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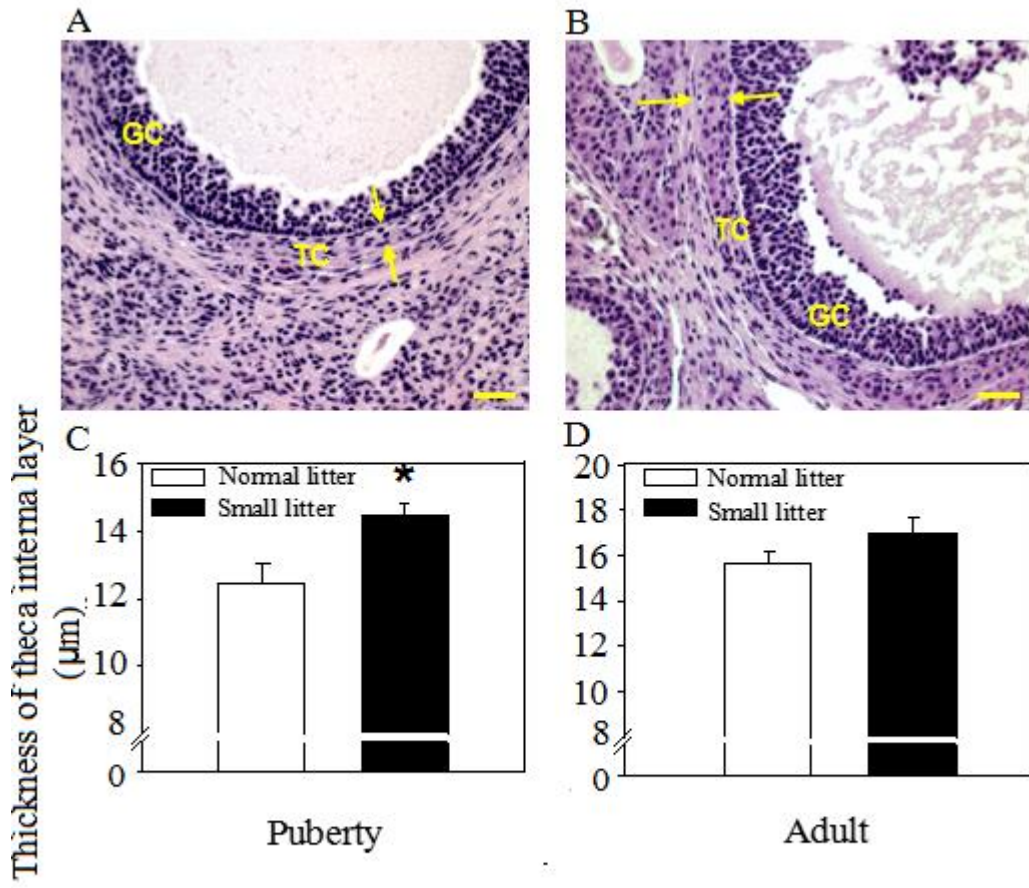
508 **Figure 1**



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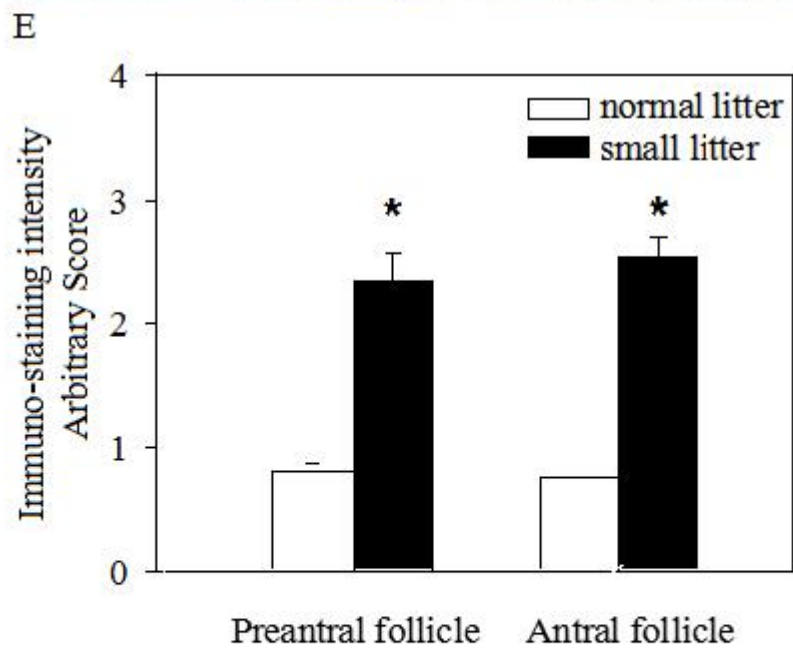
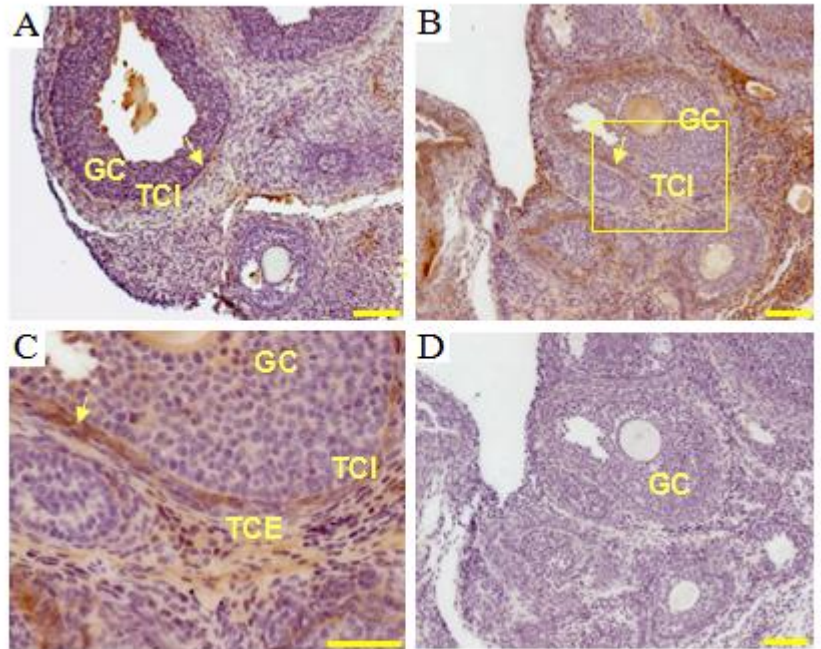




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517 **Figure 4**



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