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Expression analyses of insulin-like peptide 3, RXFP2, LH receptor and 3β-HSD in testes of normal and cryptorchid dogs

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Abstract

Insulin-like peptide 3 (INSL3) plays a key role in testicular descent in rodents, whereas in domestic animals, many aspects of the roles of INSL3 in reproductive organs after puberty are still unknown. This study was undertaken to: (1) determine the quantitative changes of gene expression of testicular INSL3, its receptor (RXFP2), LH receptor and 3β-HSD during and after puberty in normal male dogs; (2) compare the expressions of these substances in normal and cryptorchid dogs; and (3) localize the cells expressing INSL3 in normal and retained canine testes. Testes were obtained from small-breed normal male dogs (n=56) and cryptorchid dogs (n=22). Normal scrotal testes from the normal dogs (normal testes) and retained testes from both the unilateral and bilateral cryptorchid dogs (retained testes) and scrotal testes of the unilateral cryptorchid dogs (cryptorchid scrotal testes) were used. We measured the concentrations of these testicular mRNAs by quantitative real-time RT-PCR, and an enzyme immunoassay was used for measuring INSL3 peptide. Immunohistochemistry for INSL3 peptide was done in paraformaldehyde-fixed frozen testicular tissue. In the normal dogs, total amount of INSL3 mRNA per testis tended to decrease (P=0.05) from pubertal (6–12 mo) to post-pubertal (1–5 y) and decreased (P<0.01) to middle age (5–10 y), but total amount of INSL3 peptide per testis did not change among age groups. Concentrations of INSL3

mRNA were higher (P<0.01) in retained testes than those in the normal testes and cryptorchid scrotal testes, and similar differences were observed for INSL3 peptide. Reversely, total amounts of INSL3 mRNA and peptide per retained testis were lower (P<0.01) than those per normal testis, due to smaller weight of retained testes. Concentrations and total amount of RXFP2 mRNA in the retained testes were almost nil, and lower (P<0.01) than those in the normal testes and in the cryptorchid scrotal testes. Total amount of LH receptor mRNA per retained testis was lower (P<0.01) than that per normal testis. The immunohistochemical analysis revealed that INSL3 was expressed only in Leydig cells of both the normal and retained canine testes. These results suggest that INSL3 in retained testes of cryptorchid dogs is substantially expressed per unit-weight basis, but may be produced with lower amount as a whole testis. Also this study provides findings that RXFP2 gene is expressed scarcely in the retained testes, but normally in cryptorchid scrotal testes.

Keywords: INSL3; RXFP2; Leydig cell; Cryptorchid; Testis; Dog

1. Introduction

Insulin-like peptide 3 (INSL3), also known as relaxin-like factor, is a relatively newly identified peptide hormone produced by testicular Leydig cells [1–3]. Its mRNA is constitutively expressed in a differentiation-dependent manner related to the postnatal development of Leydig cell function [1, 4]. During the fetal period, INSL3 plays an important role in the trans-abdominal phase of testicular descent in mice [5, 6] and the survival of germ cells as an anti-apoptotic factor in adult humans [7] and rats [8]. INSL3 has also been suggested to have an important endocrine role in the males of many mammalian species and can readily be measured in the peripheral plasma of humans [9–11], rodents [12, 13], cattle [14] and dogs [15].

In male dogs, plasma INSL3 concentrations increased significantly from pre-pubertal to pubertal age and then declined from pubertal to post-pubertal age [15]. Lower INSL3 concentrations have been detected in bilateral cryptorchid dogs compared to normal and unilateral cryptorchid dogs, suggesting the diagnostic value of this hormone in anticipating bilaterally retained testes [15]. However, the dynamics of the expression of INSL3 at the mRNA and peptide levels associated with the development of reproductive stages remain to be elucidated.

Relaxin family peptide receptor 2 (RXFP2; formerly known as LGR8) is the

specific receptor of INSL3 [16]. RXFP2 knockout mice showed intra-abdominal cryptorchidism and male infertility due to the arrest of spermatogenesis [17, 18]. The expression of RXFP2 in adult testes was demonstrated to be localized in germ cells in seminiferous tubules and interstitial Leydig cells in humans [7], rats [7, 19] and mice [7, 20] by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. In dogs, INSL3 and RXFP2 expression were revealed by immunohistochemistry in testicular Leydig cells of both normal and cryptorchid testes, with a lack of RXFP2 expression in the genital tracts of cryptorchid testes [21]. The quantitative changes of this receptor during sexual development in canine testes have not yet been determined.

Cryptorchidism, a failure of one or both testes to descend normally into the scrotum, affects 2%–9% of newborn boys [22], 2%–8% of male horses [23] and 1.2%–10.7% of male dogs [24, 25], with a higher risk in small breeds than in larger breeds dogs [26]. INSL3/ RXFP2 signaling plays a crucial role in the process of testicular descent in mice, but differences in the testicular expressions of INSL3 and RXFP2 between normal and cryptorchid animals have not been analyzed quantitatively in any species including dog, to the best of our knowledge.

LH receptor and 3β-hydroxysteroid dehydrogenase (3β-HSD; a steroidogenic

enzyme) have also been used as a marker of testicular Leydig cells and have been identified in horses [27], rats [28] and dogs [29]. These markers were used for identifying normal and tumorous Leydig cells in dogs [29]. In equine testes, the immuno-labeling of 3β-HSD was very weak or absent in immature Leydig cells of pre-pubertal testes and increased in post-pubertal and adult testes [30]. Steroidogenesis occurs primarily in Leydig cells [31], and reduced testosterone production has been observed in cryptorchid mice, stallions and dogs [32–34]. To the best of our knowledge, there have been no studies comparing the LH receptor and 3β-HSD gene expressions among normal, scrotal and retained testes in dogs.

The objectives of the present study were to: (1) determine the quantitative changes of the gene expressions of testicular INSL3, RXFP2, LH receptor and 3β-HSD during and after puberty in normal male dogs; (2) compare the expressions of these substances in retained and scrotal testes of cryptorchid dogs with those of normal testes of normal dogs; and (3) localize cells expressing INSL3 in normal and cryptorchid canine testes.

2. Materials and methods

2.1. Animals and sampling

A total of 78 male dogs were used in the present study. The dogs were presented to a private animal clinic close to our university for ordinary contraception or treatment of cryptorchidism. All of the dogs were privately owned, and the owners' consent was obtained before the collection of samples. The study was conducted according to the regulations of the local Institutional Animal Care and Use Committee. Before surgery, testicular presence was checked manually and diagnosed as normal (n=56) if both testes were palpable inside the scrotum. Cryptorchidism was diagnosed (n=22) when one (unilateral, n=16) or both (bilateral, n=6) testes were missing in the scrotum after 6 months of age [35, 36]. All dogs belonged to small breeds, and nearly 80% were Toy Poodles, Chihuahuas, Miniature Dachshunds, Pomeranian and Shih Tzus. The ages of the dogs ranged from 6 mo to 10 y. The range of body weights was 1.4 to 8.6 kg (4.4 ± 0.2 kg; mean ± SEM). Testes samples were collected after castration or cryptorchidectomy and then immediately dispatched to the laboratory on ice. The testes were separated from the epididymides. The weight of both testes was recorded from all normal and cryptorchid dogs.

2.2. Tissue processing

Different testes samples were used for (1) a quantitative RT-PCR and enzyme

124 immunoassay (EIA) and (2) immunohistochemistry. Normal testes (either the right or
125 left testis) of the normal dogs were used for the RT-PCR and EIA (n=46) and for
126 immunohistochemistry (n=10). Retained testes of the unilateral and bilateral cryptorchid
127 dogs were used for the RT-PCR and EIA (n=19) and for immunohistochemistry (n=5).
128 Scrotal testes of the unilateral cryptorchid dogs (cryptorchid scrotal testes) were used
129 only for the RT-PCR and EIA (n=11). For the quantitative RT-PCR and EIA, testicular
130 tissue was cut into small pieces (approx. 1 cm^3) and saved at −80°C until RNA and
131 peptide extractions.
132 For the immunohistochemistry, testicular tissues were fixed overnight in 4%
133 paraformaldehyde, followed by incubating in sucrose solutions (10%, 20% and 30%)
134 for an additional 24 h at 4°C. The tissue pieces were then embedded in OCT compound
135 (Tissue-Tek, Sakura Finetek Japan, Tokyo) and maintained at −80°C until sectioning.
136
137 *2.3. RNA extraction, cDNA synthesis and real-time PCR*
138 Total RNA was isolated from a small amount of frozen testicular tissue (approx.
139 20 mg) using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the
140 manufacturer's instructions. RNA quantity and quality were evaluated using a
141 spectrophotometer (U-2000, Hitachi, Tokyo) at 260 nm. The isolated total RNA was

142 stored at −80°C until RT-PCR.

143 Table 1 lists the pairs of primers used to quantitate mRNAs for canine INSL3,
144 RXFP2, LH receptor, 3β-HSD and 18S rRNA in testicular tissue and the expected sizes
145 of their base pairs. The primers other than18S rRNA were designed based on the canine
146 nucleotide sequence registered in GenBank. The 18S rRNA primers were used as an
147 internal standard as reported [37].

148 The mRNAs were measured by reverse transcription and quantitative real-time
149 PCR with calibration curves. For the calibration of cDNA for each targeted mRNA from
150 the total RNA, an ordinary RT-PCR was performed with a Takara RNA PCR Kit (AMV)
151 Ver. 2 (Takara, Ohtsu, Japan) according to the manufacturer's instructions. The PCR
152 products were stored at −20 °C until these analyses. A portion of the PCR products was
153 electrophoresed through a 2.0% agarose gel containing 0.5 mg/mL ethidium bromide.
154 The band was dissected out on an UV transilluminator, and DNA was extracted from the
155 agarose gel, using a QIAEX II Extraction Kit (QIAGEN, Hilden, Germany). Purified
156 PCR products were sequenced directly using a sequencer (3730xl DNA Analyzer,
157 Applied Biosystems, Carlsbad, CA) by outsourcing (Bio Matrix Research, Chiba,
158 Japan).

159 The cDNA sequence data were compared with the registered sequences in

GenBank using sequence analysis software (Sequence Scanner, Applied Biosystems). All five targeted cDNA sequences were identical at 100% with the registered sequences in GenBank. The registered cDNA sequences in GenBank for INSL3, RXFP2, LH receptor, 3β-HSD and 18S rRNA are NM_001002962, NM_001005870, XM_538486, NM_001010954 and NR_046237, respectively.

The total RNA (0.5 μg) from canine testes was reverse-transcribed into cDNA using the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The reverse transcription was reacted in a Real-Time PCR System (Bio-Rad Laboratories). The subsequent real-time PCR reaction was performed using Ssofast™ EvaGreen® Supermix (Bio-Rad Laboratories) per the manufacturer's instructions. The numbers of cycle for the PCR reactions of INSL3, RXFP2, LH receptor, 3β-HSD and 18S rRNA were 40, 40, 34, 34 and 20, respectively. The concentrations of mRNA were calculated as the threshold cycle numbers of targeted mRNA for each sample divided by those of 18S rRNA. Total amount of targeted mRNA per testis was calculated from the data of mRNA concentration and testicular weight. The standards were checked for linearity in every assay with serial 10-fold diluted calibration cDNA for each targeted mRNA. The regression coefficient (R^2) value was more than 0.996 in all assays.

178

179 *2.4. Extraction of INSL3 from testicular tissue*

180 The extraction of INSL3 from testicular tissue was carried out according to the
181 procedure described earlier for bovine plasma in our laboratory [14]. First, approx. 100
182 mg of frozen testicular tissue was placed into a tube containing 500 μL of 0.1%
183 trifluoroacetic acid (TFA). Homogenization was then performed for 1 min (20 s × 3) on
184 ice using a Polytron homogenizer (Kinematica, Littau, Switzerland). Another tube with
185 300 μL of acetonitrile was kept ready in advance, into which 500 μL of the
186 homogenized mixture was transferred immediately after homogenization. This was then
187 kept at 4°C for overnight after mixing by vortexing. Next, the mixture was centrifuged
188 at 15,000 × g for 10 min at 4°C. The resulting supernatant was then transferred into
189 another tube and concentrated by a vacuum centrifugation (Centrifugal Concentrator
190 CC-105; Tomy Seiko, Tokyo) for approx. 3 h (final volume, approx. 60 μL). Finally,
191 450 μL of 0.05 M phosphate buffer (pH 7.5) was added to the concentrated supernatant,
192 which was stored at −30°C until the assay.

193

194 *2.6. INSL3 assay*

195 The concentrations of INSL3 peptide were determined using an EIA. The

196 immunoassay procedure was basically similar to the previously described time-resolved
197 fluorescence immunoassay (TRFIA) [15], except that biotinylated canine INSL3 was
198 used for the EIA instead of europium-labeled human INSL3. Briefly, eight-well strips
199 were coated with 100 μL of anti-mouse IgG antibody (MP Biochemicals, Solon, OH; 5
200 μg/mL in 0.05M sodium bicarbonate; pH 9.7), and nonspecific binding sites were
201 blocked overnight with assay buffer containing 2% bovine serum albumin (BSA; Cohn
202 Fraction V, Sigma-Aldrich, St. Louis, MO), and 0.02% ProClin 950 (Sigma-Aldrich) in
203 0.01M PBS, pH 7.4.

204 Next, 50 μL of canine INSL3 standard [15] or sample medium and 50 μL of anti-
205 bovine INSL3 mouse monoclonal antibody (2-8F [14, 15]; 1:1,000,000 dilution in assay
206 buffer) were dispensed and incubated for 2 h at room temperature. After that, 50 μL of
207 biotinylated canine INSL3 (2 ng/mL in assay buffer) was added and incubated for a
208 further 1 h. The biotinylated canine INSL3 was synthesized by the same procedure used
209 for the biotinylated bovine INSL3 [14]. The wells were then washed three times with
210 saline containing 0.05% Tween 20 and incubated for 30 min with horseradish
211 peroxidase-labeled streptavidin (KPL, Gaithersburg, MD; 100 ng/mL in assay buffer).
212 The wells were then again washed three times with saline containing 0.05% Tween 20
213 and incubated for another 30 min at room temperature with 100 μL substrate solution

214 containing 3,3`,5,5`-tetramethylbenzidine (TMB). The reaction was stopped by adding
215 50 μL of 2 M sulfuric acid, and the optical density was measured at 450 nm using an
216 xMark microplate absorbance spectrophotometer (Bio-Rad Laboratories). The assay
217 detection range was from 0.05 to 10 ng/mL. The intra- and inter-assay coefficients of
218 variation were 14.7% and 16.2%, respectively. The hormonal specificity of the anti-
219 bovine INSL3 antibody (2-8F) was validated previously [14]. The INSL3 peptide
220 concentrations for each sample were normalized by protein amount in the homogenate.
221 The protein amount was measured by BCA Protein Assay Reagent Kit (Thermo
222 Scientific, Rockford, IL). Total amount of INSL3 peptide per testis was calculated from
223 the data of INSL3 concentration and testicular weight.
224
225 *2.6. Immunohistochemistry*
226 Testicular tissues were examined by immunohistochemistry to check the
227 expression of INSL3 peptide. Briefly, sections were cut from OCT-embedded tissue
228 using a Cryostat (Leica CM1510S, Leica Microsystems, Wetzlar, Germany) at 7 μm and
229 attached on glass slides (Platinum, Matsunami Glass, Osaka, Japan) treated with an anti-
230 stripping reagent. The slide glasses were then immersed in a bottle containing PBS for
231 washing, and the washing was repeated by transferring the slides into second and third

washing bottles.

ImmPRESS™ Reagent Kit Peroxidase Anti-Mouse Ig and the Peroxidase Substrate Kit DAB (Vector Laboratories, Burlingame, CA) were used for the immunohistochemistry. Each slide was then blocked with 250 µL of 2.5% normal horse serum and incubated for 20 min. After the blocking solution was discarded, the sections were incubated overnight with the primary antibody (Anti-bovine INSL3 antibody [2-8F]; 1: 1000 dilutions). After incubation with the primary antibody, the slides were incubated in 0.3% H_2O_2 for 30 min for quenching endogenous peroxidases. Thereafter, 350 µL secondary antibody (Anti-mouse Ig) was applied on slides and left to stand for 30 min. Finally, 380 µL of DAB solution was applied on the slides and the reaction was stopped after approx. 5–10 min. All incubations were carried out at room temperature in a humidified chamber except for those with primary antibody (4°C).

Following the incubation with primary or secondary antibody or 0.3 % H_2O_2, the sections were washed (3 × 5 min) in 0.01 M PBS solution (pH 7.4). Staining with Hematoxylin was done for the same testes specimens used for immunohistochemistry in a different slide to check the cellular structures of the normal and retained testes including the presence/absence of sperm. The specificity of the staining with anti-bovine INSL3 antibody was confirmed in parallel sections by using assay buffer instead

of primary antibody, which was considered the negative control for the specificity of the INSL3 immunostaining.

2.7. Data analyses

To evaluate the mRNA and peptide changes with the age, we categorized the normal dogs (6 mo–10 y; n=46) into pubertal (6 mo–1 y; n=19), post-pubertal (1–5 y; n=17) and middle age (5–10 y; n=10). Samples obtained from pubertal and post-pubertal ages (n=36) were used (samples from the middle age were excluded) for the comparison among the normal, retained and cryptorchid scrotal testes groups, because all of the cryptorchid dogs were within the age range from pubertal to post-pubertal age. Immunohistochemistry was done in pubertal (n=3), post-pubertal (n=4) and middle-age (n=3) normal dogs and cryptorchid dogs (n=5).

We also categorized the normal dogs' breeds into four groups: (1) Toy Poodles (n=12), (2) Miniature Dachshunds (n=10), (3) Chihuahuas (n=7), and (4) others (n=17), to compare breed differences by using the total testicular weight per body weight as a parameter. The normal dogs that were used to monitor age-related quantitative changes of mRNAs and the INSL3 peptide were analyzed, not the dogs used for the immunohistochemistry. We used the Chi-square test to identify any differences in the

breed distribution between the normal (n=56) and cryptorchid (n=22) dogs.

The evaluations of INSL3, RXFP2, LH receptor, 3β-HSD mRNAs and INSL3 peptide were performed by a two-way analysis of variance (ANOVA) using generalized linear (GENLIN) models of SPSS version 22 software (IBM, Somers, NY) to assess the effects of age and the testicular status of the animal (normal, retained testes or cryptorchid scrotal testes). Differences in mRNAs and peptides among the various age groups were compared using pairwise comparisons of the GENLIN procedure by the least significant difference (LSD) post hoc test. Data are expressed as mean ± SEM. Differences were considered significant at $P<0.05$.

3. Results

The mean testicular weight increased significantly ($P<0.05$) from the pubertal (2.40 ± 0.40 g) to the post-pubertal age (3.46 ± 0.29 g), and did not change from the post-pubertal to the middle-age (3.72 ± 0.47 g) dogs. The testicular weight of the retained testes of the cryptorchid dogs (0.56 ± 0.06 g) was much lower ($P<0.01$) than the scrotal testes (2.02 ± 0.41 g) of the unilateral cryptorchid dogs and the normal testes (2.90 ± 0.26 g) of the normal dogs. The testicular weight of the scrotal testes tended to be lower ($P=0.07$) than that of the normal testes. There was no significant difference in

286 the testicular weight per body weight values among the various breeds (Toy Poodles,
287 1.09 ± 0.11; Miniature Dachshunds 1.48 ± 0.19; Chihuahuas 1.06 ± 0.21; others 1.20 ±
288 0.08) of normal dogs used for the analyses of age-related changes of mRNAs and
289 INSL3 peptide concentrations. The distributions of breeds were also the same between
290 the normal and cryptorchid dogs (data not shown).

291 The INSL3 mRNA concentrations decreased ($P<0.05$) from pubertal to post-
292 pubertal and from post-pubertal to middle age (Fig 1A), whereas the INSL3 peptide
293 concentrations did not differ significantly among the age groups (Fig. 1B). Total amount
294 of INSL3 mRNA per testis tended to decrease ($P=0.05$) from pubertal to post-pubertal
295 age and decreased ($P<0.05$) from pubertal to middle age (Fig. 1E). However, total
296 amount of INSL3 peptide per testis did not differ significantly among the age groups
297 (Fig. 1F). The RXFP2 mRNA concentrations increased significantly ($P<0.01$) from
298 pubertal to post-pubertal age, but there was no difference between post-pubertal and
299 middle age (Fig. 1C). Total amount of RXFP2 mRNA per testis did not change among
300 the age groups (data not shown).

301 The LH receptor mRNA concentrations did not differ between pubertal and post-
302 pubertal age in the normal dogs, but they decreased significantly ($P<0.01$) from pubertal
303 to middle age (Fig. 1D). The concentrations of 3β-HSD mRNA did not differ

304 significantly among age groups in normal dogs (data not shown). Total amount of LH
305 receptor and 3β-HSD mRNAs per testis did not change among the age groups (data not
306 shown).

307 The INSL3 mRNA concentrations were significantly higher ($P<0.01$) in the
308 retained testes of the cryptorchid dogs compared to the normal testes of the normal dogs
309 and the scrotal testes of the unilateral cryptorchid dogs (Fig. 2A). A very similar INSL3
310 mRNA concentration was observed between the normal testes of normal dogs and the
311 scrotal testes of unilateral cryptorchid dogs (Fig. 2A). The total amount of INSL3
312 mRNA per retained testis was significantly lower ($P<0.01$) than that per normal testis
313 and did not differ significantly from that per cryptorchid scrotal testis (Fig. 2E). The
314 INSL3 peptide concentrations in the retained testes were significantly higher ($P<0.05$)
315 than those in the cryptorchid scrotal testes and tended to be higher ($P=0.08$) than those
316 in the normal testes (Fig. 2B). The INSL3 peptide concentrations for the scrotal testes of
317 the unilateral cryptorchid dogs did not differ from the normal testes of the normal dogs
318 (Fig. 2B). The total amount of INSL3 peptide per retained testis was significantly lower
319 ($P<0.01$) than that per normal testis, but did not differ significantly from that per
320 cryptorchid scrotal testis (Fig. 2F). The total amount of INSL3 peptide per cryptorchid
321 scrotal testis tended to be lower ($P=0.06$) than that per normal testis (Fig. 2F).

322 The RXFP2 mRNA concentrations were almost negligible in the retained testes,
323 and were much lower ($P<0.001$) than those in the normal testes (Fig. 2C). A similar
324 concentration of RXFP2 mRNA was observed between the normal testes of the normal
325 dogs and the scrotal testes of the unilateral cryptorchid dogs (Fig. 2C). The total amount
326 of RXFP2 mRNA per retained testis was almost nil and much lower than that per
327 normal testis ($P<0.001$) and cryptorchid scrotal testis ($P<0.05$; Fig. 2G). The total
328 amount of RXFP2 mRNA per cryptorchid scrotal testis tended to be lower ($P=0.08$)
329 than that per normal testis (Fig. 2G).
330 The LH receptor mRNA concentrations did not differ between the normal and
331 cryptorchid dogs (data not shown). Total amount of LH receptor mRNA per retained
332 testis was lower ($P<0.01$) than that per normal testis, and tended to be lower ($P=0.14$)
333 than that per cryptorchid scrotal testis (Fig. 2H). Significantly higher ($P<0.01$)
334 concentrations of 3β-HSD mRNA were observed in the retained and scrotal testes of the
335 cryptorchid dogs compared to the normal testes of the normal dogs (Fig. 2D). Total
336 amount of 3β-HSD mRNA per retained testis did not differ significantly among normal,
337 retained and cryptorchid scrotal testes (data not shown).
338 We performed immunohistochemistry to examine the specific cell type(s) that
339 shows INSL3 peptide expression in various age groups of normal testes of normal dogs

and retained testes of cryptorchid dogs. Only Leydig cells of both the normal (pubertal, post-pubertal and middle age) and retained testes were immune-reactive to INSL3 antibody (shown in supplemental Fig. 1). The size of the seminiferous tubules per testicular area seemed to increase from pubertal to post-pubertal and middle age.

The intensity of staining for INSL3 was clearly stronger in the Leydig cells of the retained testes compared to those of the normal testes in all age categories. No other testicular cell showed any immune reaction for INSL3 antibody (shown in supplemental Fig. 1). When the primary antibody was omitted, no immunostaining was observed (shown in supplemental Fig. 1). The Hematoxylin staining revealed the presence of sperm inside seminiferous tubules in the testes of the normal dogs (pubertal, post-pubertal and middle age) but the absence of sperm in the retained testes of the cryptorchid dogs (data not shown).

4. Discussion

It was reported that in rodents, INSL3 has pivotal roles in testicular descent in the fetal period [5, 6]. A role of INSL3 in the reproductive organs of domestic animals after puberty has rarely been reported. The changes of testicular INSL3 and its receptor, RXFP2, in pubertal and post-pubertal normal male animals have not yet been

358 elucidated, and a quantitative comparison of the testicular INSL3-receptor system
359 between cryptorchid and normal animals has not been reported. In this study, we
360 examined the gene expressions of INSL3 and RXFP2 in testes during puberty, post-
361 puberty and middle age in normal dogs to elucidate the changing pattern of these genes'
362 expression with age and sexual maturity. We also compared the INSL3 and RXFP2 gene
363 expressions in retained and scrotal testes of cryptorchid dogs with those of normal testes
364 of normal dogs. This is apparently the first study regarding quantitative changes of
365 testicular INSL3 and RXFP2 gene expression with age and sexual maturity and the
366 comparison of these expressions between normal and cryptorchid dogs.

367 The present results revealed that total amount of INSL3 mRNA per testis
368 decreased by aging in normal dogs despite increase of testicular weight, although the
369 amount of INSL3 peptide per testis did not change significantly during the same ages.
370 These results may indicate that the transcriptional activity of the gene encoding INSL3
371 in canine testes is reduced by the aging, but such a change is not reflected in the peptide
372 content. It was suggested that INSL3 concentrations in peripheral blood are higher in
373 the pubertal age and decline in the post-pubertal age in male dogs [15]. The change of
374 INSL3 mRNA amount per testis from puberty to middle age observed in the present
375 study, but not of the peptide, is likely to correspond to those of INSL3 concentrations in

blood. The reasons for the inconsistency of changes between testicular INSL3 mRNA and the peptide amount around canine puberty observed in our study are unknown. In male rats, the INSL3 concentrations in plasma transiently increased during puberty and decreased after puberty [13]. Testicular INSL3 mRNA concentrations in rats of advanced age (22–24 mo) were reduced compared to post-pubertal age (3 mo) [38]. In the present study, we did not examine histological changes of testicular cellular components including Leydig cells and various stages of germ cells during aging in the same samples which were measured for mRNAs and INSL3 peptide. Clearly, further studies are required to elucidate changes of INSL3 expression level per Leydig cell basis during puberty and aging in dogs.

We found higher mRNA and slightly increased peptide concentrations of INSL3 in the retained testes of the cryptorchid dogs compared to the normal testes of the normal dogs in the present study. Our immunohistochemistry data also showed that the areas occupied by INSL3-producing Leydig cells per a certain area of testicular tissue seem larger in the retained testes than in the normal testes, but we did not perform quantitative analyses of INSL3-producing Leydig cells in normal and retained testes in this study. However, total amounts of INSL3 mRNA and peptide per testis were reduced in the retained testes relative to normal testes due to much smaller size of the former,

suggesting that the canine retained testis may produce lower INSL3 as a whole testis. It has been suggested that INSL3 secretion in bilateral cryptorchid dogs is reduced compared to the normal and unilateral cryptorchid dogs [15]. The current study also shows that concentrations and total amount of INSL3 mRNA and peptide are similar between the scrotal testes of unilateral cryptorchid dogs and the normal testes of normal dogs. These results may be accorded with the previous findings that plasma INSL3 concentrations are similar between normal and unilateral cryptorchid dogs [15].

The present study provides findings that the gene expression of RXFP2 is almost disappeared in canine retained testes at both of per unit-weight basis and per whole-testis basis, in marked contrast to the higher expression of the receptor in normal testes. A previous histological examination also showed a lack of RXFP2 immunoreactivity in the genital tracts of cryptorchid canine testes [21]. Thus, it is likely in cryptorchid dogs that the substantial amount of INSL3 secreted in a retained testis cannot transduce its signal to cells within the testis, although we did not measure protein levels of RXFP2 receptor.

We speculate that the drastic reduction of RXFP2 mRNA in the retained testes may be caused mainly by the absence of advanced stages of germ cells that express RXFP2, due to impaired spermatogenesis [7, 19, 20]. It is also plausible that the down-

412 regulation of RXFP2 in Leydig cells by an autocrine mechanism [39, 40] with high or
413 substantial concentrations of INSL3 could partly contribute to the loss of RXFP2 gene
414 expression since relatively plenty of Leydig cells exist in the retained testes. It remains
415 to be determined in future studies whether the down-regulation of RXFP2 occurs in
416 Leydig cells in canine retained testes. We observed that the scrotal testes of unilateral
417 cryptorchid dogs exhibit RXFP2 expression similar to that of the normal testes of
418 normal dogs, implying that the lack of the receptor gene expression in the retained testes
419 probably occurs as a consequence of — not as a cause of — the retention of the testes.
420 In addition to INSL3 and RXFP2, we analyzed the gene expression of LH
421 receptor and 3β-HSD, which are also known as markers of Leydig cells [3, 27–29],
422 during the course of puberty in the testes of the normal and cryptorchid dogs. Our
423 findings revealed that mRNAs for both LH receptor and 3β-HSD showed differential
424 dynamics compared with INSL3 mRNA during puberty and in the testes of the
425 cryptorchid dogs. We speculate that the regulatory mechanisms for the gene expressions
426 of these three markers for Leydig cells differ. It is not clear why the concentrations of
427 3β-HSD mRNA were increased not only in the retained but also in the scrotal testes of
428 unilateral cryptorchid dogs in the present study. There could be a mechanism in
429 unilateral cryptorchid dogs in which a retained testis may affect the function of the other

scrotal testis through substances secreted from the retained testis [34]. The region of canine LH receptor mRNA selected for the real-time PCR in this study is known to encode the receptor protein, but the transcript may slightly include splicing variants which encode non-functional LH receptors. Thus it should be noted that not all of the mRNA would be expressed as the functional LH receptor.

In conclusion, higher INSL3 mRNA per unit-weight basis and clear staining of Leydig cells for INSL3 peptide in the retained testes of cryptorchid dogs indicate the substantial expression of INSL3 in Leydig cells of the retained testes. However, smaller amount of INSL3 is likely to be produced per a whole retained testis due to its diminutive size. Also the present study reveals that RXFP2 gene expression is lost in the retained testes, but occurs normally in cryptorchid scrotal testes.

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566 **Table 1.** Oligonucleotide sequences of the primers used for real-time PCR, their

567 location and the product sizes expected in canines

Primer	**Primer sequence (5'–3')**	**Location**	**Product length** (bp)
INSL3	F: GGGGGCCCGCGCTGGTCCTC	145–164	181
	R: CAGCTGCTCGCCGGTGGTGGTGATG	325–301	
RXFP2	F: CAACTCACGCTACATCCATCAAAAT	1292–1316	190
	R: AGGACGGACACTTCAGTAGACAGC	1481–1458	
LH receptor	F: TGTGGTGGCCTTCATCATCATTTG	1632–1655	346
	R: AAGTTCAGCCCGACGTTTACAGC	1977–1955	
3β-HSD	F: CAGAATGCCCACGAAGAAGAG	541–561	259
	R: AGACGGGGTTGACTATGGAGAA	799–778	
18S rRNA	F: TGGTTGATCCTGCCAGTAGCA	5–25	96
	R: ATGAGCCATTCGCAGTTTCACT	100–79	

568

Figure legends

Fig. 1. Changes in testicular concentrations of INSL3 mRNA (A), INSL3 peptide (B), RXFP2 mRNA (C), LH receptor mRNA (D), and total amount per testis of INSL3 mRNA (E) and INSL3 peptide (F) in various age groups of normal male dogs. Results are shown for pubertal (6–12 mo, n=19), post-pubertal (1–5 y, n=17) and middle age (5–10 y, n=10). Data are mean ± SEM. $^{a–c}$Values without a common superscript differed significantly for A and E ($P<0.05$), and for C and D ($P<0.01$).

Fig. 2. Testicular concentrations of INSL3 mRNA (A), INSL3 peptide (B), RXFP2 mRNA (C), 3β-HSD mRNA (D), and total amount per testis of INSL3 mRNA (E), INSL3 peptide (F), Rxfp2 mRNA (G) and LH receptor mRNA (H) in normal (n=36), retained (n=19) and scrotal testes (n=11). Data are mean ± SEM. ^{a,b}Values without a common superscript differed significantly for B ($P<0.05$), for A, D, E, F and H ($P<0.01$) and for C and G ($P<0.001$).

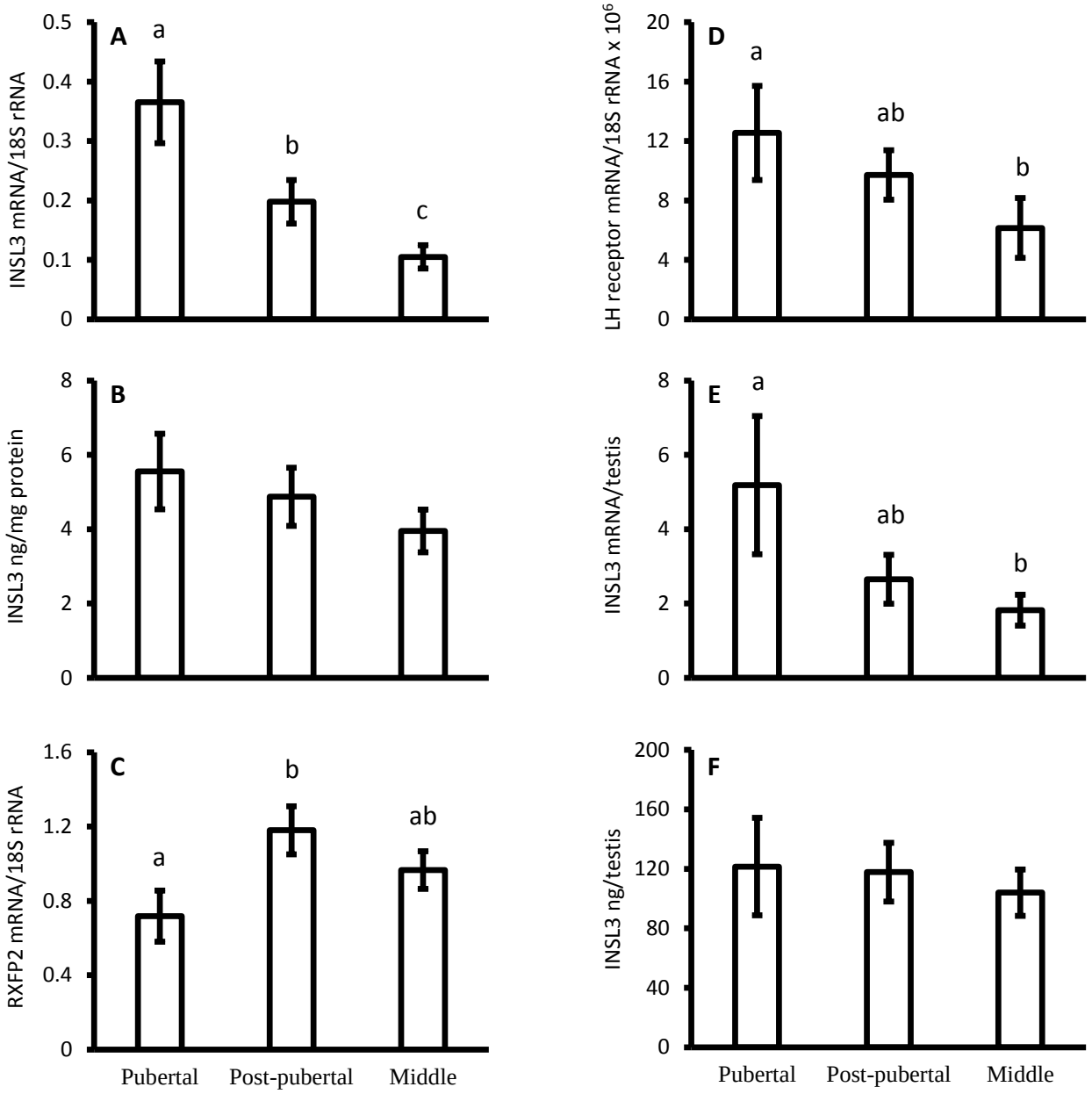

A
a
b
c
INSL3 mRNA/18S rRNA
0.5
0.4
0.3
0.2
0.1
0
D
a
ab
b
LH receptor mRNA/18S rRNA x 10^6
20
16
12
8
4
0
B
INSL3 ng/mg protein
8
6
4
2
0
E
a
ab
b
INSL3 mRNA/testis
8
6
4
2
0
C
a
b
ab
RXFP2 mRNA/18S rRNA
1.6
1.2
0.8
0.4
0
F
INSL3 ng/testis
200
160
120
80
40
0
Pubertal
Post-pubertal
Middle
Pubertal
Post-pubertal
Middle

Fig. 1

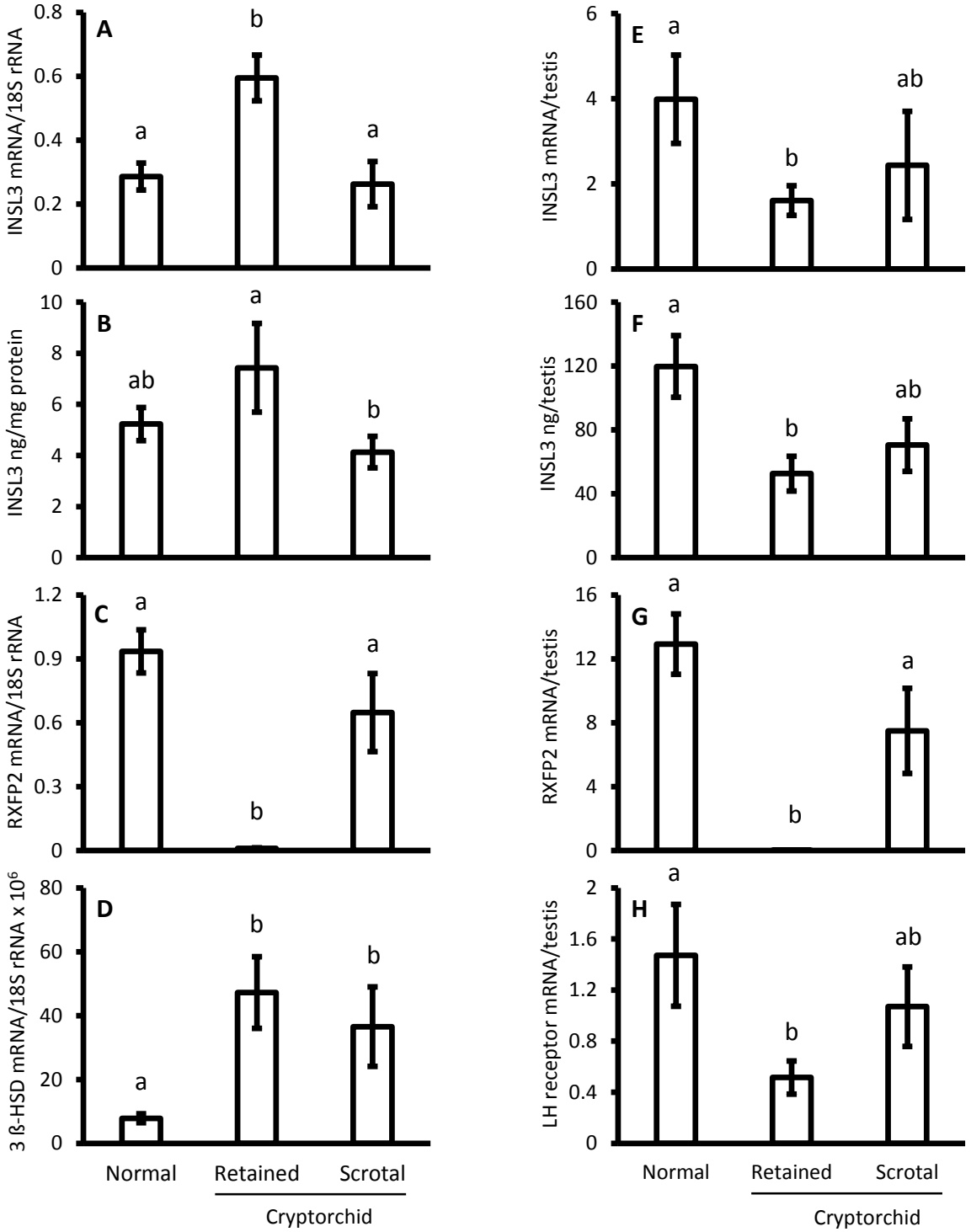
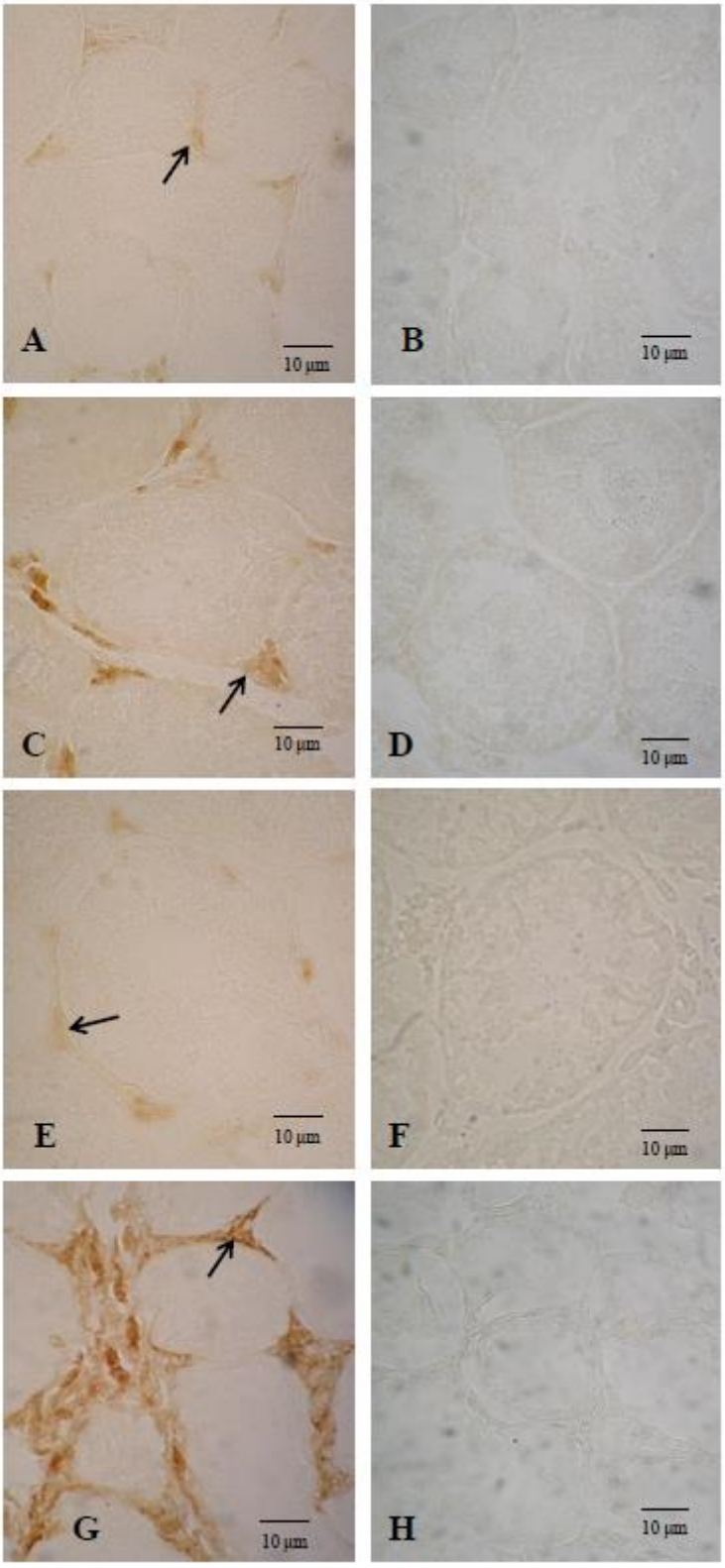

A
INSL3 mRNA/18S rRNA
0.8
0.6
0.4
0.2
0
a
b
a
B
INSL3 ng/mg protein
10
8
6
4
2
0
ab
a
b
C
RXFP2 mRNA/18S rRNA
1.2
0.9
0.6
0.3
0
a
b
a
D
3 ß-HSD mRNA/18S rRNA x 10⁶
80
60
40
20
0
a
b
b
E
INSL3 mRNA/testis
6
4
2
0
a
b
ab
F
INSL3 ng/testis
160
120
80
40
0
a
b
ab
G
RXFP2 mRNA/testis
16
12
8
4
0
a
b
a
H
LH receptor mRNA/testis
2
1.6
1.2
0.8
0.4
0
a
b
ab
Normal
Retained
Scrotal
Cryptorchid
Normal
Retained
Scrotal
Cryptorchid

Fig. 2



Supplemental data Fig. 1

Supplemental data

Fig. 1. Representative photomicrographs of the immunohistochemical staining of INSL3 peptide (brown staining) in canine normal (A, pubertal; C, post-pubertal; E, middle age) and retained (G) testes. In both the normal and retained testes, only testicular Leydig cells (black arrows) showed INSL3 immunolabeling. The staining intensity was stronger in the retained testes compared to the normal testes in all age groups. When the primary antibody was omitted, immunolabeling was not observed in the normal (B, pubertal; D, post-pubertal; F, middle age) or retained (H) testes.