

Expression and localization of epidermal growth factor, transforming growth factor- α and epidermal growth factor receptor in the canine testis

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Abstract. Gene expression of epidermal growth factor (EGF), transforming growth factor- α (TGF- α) and EGF receptor (EGF-R) and the localization of the corresponding proteins in the canine testis were studied. Levels of mRNA expressions were determined by semiquantitative reverse transcription polymerase chain reaction in the testes of the peripubertal (4–6 months), young adult (3–4 years), advanced adult (7–8 years) and senescent (11–16 years) groups. The EGF-R mRNA level in the testes of the peripubertal group was significantly higher than those in the other groups, whereas there was no difference in EGF and TGF- α mRNA levels among groups. Immunohistochemical stainings for EGF, TGF- α and EGF-R in the testis revealed that immunoreactivity in the seminiferous epithelium and Sertoli cell was weak and nonspecific for the stage of spermatogenesis, and distinct staining was found in Leydig cells. These results suggest that the EGF family of growth factors may be involved in testicular maturation and function in the dog.

Key words: Dog, Epidermal growth factor (EGF), EGF receptor (EGF-R), Testis, Transforming growth factor- α (TGF- α)
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Epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) belong to the EGF family of growth factors, which also includes amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin, epiregulin, heregulins/neu differentiation factors, Schwannoma-derived growth factor and vaccinia virus growth factor. These ligands bind to the receptor subtypes of the ErbB gene family, which is comprised of four receptor tyrosine kinases: ErbB1 (EGF-R), ErbB2, ErbB3 and ErbB4. EGF and TGF- α can interact with EGF-R via homodimerization or heterodimerization [1].

The expression and localization of EGF family members and EGF-R in the testis have been studied in the rat [2–4], mouse [2, 5], boar [6], bull [7], monkey [3, 8] and human being [9, 10]. The production site and protein localization for these factors have been shown to be different among species. It is reported that TGF- α expressed in the rat testis stimulates development of the fetal testis [4, 11] and proliferation of Sertoli cells [12]. EGF, TGF- α and betacellulin all stimulate spermatogonial proliferation *in vitro* in rats [13], and TGF- α has been shown to promote differentiation of type A spermatogonia in the mouse cryptorchid testis [14]. In

relation to endocrinological function, EGF increases testosterone production in Leydig cells [15–17] and inhibits aromatase activity in Sertoli cells [18]. Although accumulating evidence suggests that the EGF family of growth factors plays important roles in the testis in some mammalian species, even the expression and localization of EGF family members and their receptors have not been studied in the canine testis.

This study examined the gene expression of EGF, TGF- α and EGF-R and localization of the corresponding proteins in the canine testis and their age-related changes.

Materials and Methods

Animals

All testicular tissues were obtained from dogs referred to the veterinary clinic of Osaka Prefecture University and other animal clinics outside the university for routine castration, and no animal was operated upon or killed specifically for this study. Some dogs had prostatic hyperplasia, perineal hernia, inguinal hernia or perianal gland adenoma. Normal testes of the dogs without cryptorchidism or testicular abnormality were used in this study. Table 1 shows the ages, body weights and breeds of the dogs and disorders. The epididymis was removed from the testis, and a part of the testis from some dogs, including the middle portion, was frozen in liquid nitrogen and stored at -90°C for RNA extraction. Another part of the tissue from the same dogs or a part of the testes from the other dogs was fixed in Bouin's fluid for immunohistochemical study.

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Table 1. Ages, body weights and breeds of the dogs used in the experiment

Case no.	Age	Body weight (kg)	Breed
1	4 months	7.5	Cocker spaniel
2	4 months	4.2	Shih Tzu
3	5 months	10.2	Labrador retriever
4	5 months	11.3	Mix
5	5 months	2.0	Chihuahua
6	5 months	3.1	Toy poodle
7	5 months	3.3	Maltese
8	6 months	5.2	Shih Tzu
9	1 year	31.0	Golden retriever
10	1 year	3.9	Papillon
11	1 year	28.2	Golden retriever
12	3 years	4.8	Shih Tzu
13	3 years	5.5	Shih Tzu
14	3 years	6.6	Cocker spaniel
15	3 years	5.8	Shih Tzu ^a
16	4 years	3.1	Pomeranian
17	7 years	2.8	Pomeranian
18	7 years	9.6	Shiba
19	7 years	12.3	Boston terrier
20	7 years	13.4	Shetland Sheepdog ^b
21	8 years	7.1	Shih Tzu ^c
22	11 years	13.9	Cocker spaniel ^d
23	11 years	12.1	Mix ^b
24	14 years	16.8	Mix ^d
25	16 years	18.0	Unknown ^d

^a The dog had prostatic hyperplasia, ^b perineal hernia, ^c inguinal hernia, or ^d perianal gland adenoma.

RT-PCR

Expression of mRNAs for EGF, TGF- α and EGF-R was determined by reverse transcription polymerase chain reaction (RT-PCR), and their relative expression was measured using 18S rRNA as an internal standard. Four groups were created according to the ages of the dogs. The case numbers of the dogs used for RNA extraction were 1, 2, 3, 4 and 8 for the peripubertal (4–6 months of age) group; 12, 13, 14, 15 and 16 for the young adult (3–4 years of age) group; 17, 18, 19, 20 and 21 for the advanced adult (7–8 years of age) group; and 22, 23 and 25 for the senescent (11–16 years of age) group.

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan), quantified by UV absorption measurements and stored at -90°C . Isolated RNA was reverse transcribed into cDNA using a Takara RNA PCR Kit (AMV Ver.2) (Takara, Ohtsu, Japan) and random 9-mer oligonucleotides (Takara). The cDNA samples, equivalent to approximately 250 ng of total RNA, were then used for PCR. To ascertain that cDNA was not contaminated with genomic DNA, the reverse transcription procedure for each sample was also performed in the absence of the reverse transcriptase. The reaction mixture including random 9-mer oligonucleotides was preincubated for 10 min at 30°C prior to cDNA synthesis. The reverse transcription reactions were carried out at 45°C for 30 min, heated to 99°C for 5 min to terminate the reaction and cooled to 5°C for 5 min using a MiniCycler (MJ Research, Watertown, MA, USA). The reaction

mixture was stored at -20°C . After the reverse transcription reaction, PCR amplification was performed with 200 nM primers. The sequences of the primer pairs that could detect canine EGF and EGF-R were the same as those reported by Kida *et al.* [19], and those of the primer pairs that could detect canine TGF- α and 18S rRNA were the same as those reported by Tamada *et al.* [20] and Hatoya *et al.* [21], respectively. The sequences of oligonucleotide primers and predicted cDNA sizes were as follows: 5'-TGGCCAGTGGGAATAACGAT, sense, and 5'-TGCCAATGGATGAACTACAACC (288bp), antisense, for EGF; 5'-GGAGGGCGCTGGGCTTCTCG, sense, and 5'-GGCCTTGAGAAACAGCACGTCC (377bp), antisense, for TGF- α ; 5'-TACAGCTTTGGTGCCACCTG, sense, 5'-GGCCAAGCCTGAATCAGCAA (380bp), antisense, for EGF-R; and 5'-CCGGTACCTGGTTGATCCTGCCAGTAGCA, sense, 5'-CCGGATCCATGAGCCATTCGCAGTTTCACT (112bp), antisense, for 18S rRNA. The PCR conditions were 2 min at 94°C for denaturation, followed by the specified number of cycles of 30 sec at 94°C , 30 sec at 66°C and 90 sec at 72°C . In preliminary experiments, the amount of total RNA and PCR cycles used in this semiquantitative RT-PCR was determined by estimating the linear portion of the RT-PCR products using increasing amounts of total RNA and increasing numbers of cycles. The desired expression level of both products of target genes and internal controls in the same sample was obtained by delaying the addition of the primer pairs for internal controls by 23 (EGF), 17 (TGF- α) and 20 (EGF-R) cycles, respectively. The RT-PCR products were electrophoresed, and the bands of targets and internal controls were scanned and analyzed using the NIH Image 1.61 software (National Institutes of Health, Bethesda, MD, USA). Each PCR was performed in duplicate. The densitometric intensities of targets were normalized to that of each internal control. The specificity of the amplified products was checked by restriction enzyme analysis.

Immunohistochemistry

Immunohistochemical staining for EGF, TGF- α and EGF-R and hematoxylin and eosin (HE) staining were performed in the testes of 5-month-old dogs (case nos. 5, 6 and 7), 1-year-old dogs (case nos. 9, 10 and 11), 3-year-old dogs (case nos. 12 and 13) and a 14-year-old dog (case no. 24). Immunohistochemical staining of these proteins was performed as described previously [19, 20], using reagents from a Zymed Histostain-SP kit (Zymed Laboratories, San Francisco, CA, USA). In summary, paraffin-embedded tissue blocks were cut into 7- μm sections. The sections were deparaffinized, hydrated in phosphate-buffered saline (PBS) for 20 min and then incubated in blocking solution (10% normal goat serum for EGF and TGF- α , Zymed Laboratories; 10% normal rabbit serum for EGF-R, Cedarlane Laboratories, Hornby, ON, Canada) for 10 min prior to incubation with a primary antibody for 24 h at 4°C . For EGF, a rabbit antiserum raised against rat EGF (IgG, Nashville, TN, USA) was used at 300-fold dilution. For TGF- α , a mouse monoclonal antibody (IgG) raised against the peptide (34–43) of human TGF- α (Biogenesis, DA, UK) was used at a concentration of $29.5\ \mu\text{g}/\text{ml}$. For EGF-R, a sheep polyclonal antibody (IgG) raised against the fusion protein containing a partial sequence of the cytoplasmic domain of the human EGF-R (Upstate Biotechnology, Lake Placid, NY, USA) was used at a concentration of $20\ \mu\text{g}/\text{ml}$. Briefly, the method involves

biotinylated secondary antibodies obtained by immunizing goats with rabbit IgG (for EGF) or mouse IgG (for TGF- α) and by immunizing rabbits with sheep IgG (for EGF-R), a horseradish peroxidase-streptavidin conjugate, and a substrate chromogen mixture. Blocking of endogenous peroxidase activity was achieved by incubation for 45 sec in 0.23% periodic acid (Wako Pure Chemical Industries, Osaka, Japan) in PBS after the secondary antibody incubation. A red reaction product produced by 3-amino-9-ethylcarbazole (AEC) indicated the sites of immunostaining. Staining was repeated at least three times for each block. The specificity of each antibody was tested immunohistochemically by incubation of PBS instead of the primary antibodies or preabsorption of the primary antibodies with rat EGF (IgG) at a concentration of 50 μ g/ml, human TGF- α (Strathmann, Hamburg, Germany) at a concentration of 167 μ g/ml and human EGF-R (Upstate Biotechnology) at a concentration of 800 μ g/ml, respectively.

Statistics

Differences in EGF, TGF- α , and EGF-R mRNA expression between groups were evaluated using ANOVA, followed by Fisher's protected least significant difference post hoc analysis (Statcel, the add-in forms on Excel, 1st ed.; OMS, Tokorozawa, Japan). The level of significance was set at $P < 0.05$. All values are expressed as the mean \pm SEM.

Results

Expression of EGF, TGF- α and EGF-R mRNA in the canine testis

With primers capable of detecting canine EGF, TGF- α and EGF-R mRNAs, one distinct band was observed as expected in all RNA samples extracted from the canine testis. The levels of expressions in the different groups, which were determined by semiquantitative RT-PCR, are shown in Fig. 1. The levels of EGF mRNA and TGF- α mRNA expressions were not different among groups. The level of EGF-R mRNA expression in the testes of the peripubertal group was significantly higher than those in the other groups.

Immunohistochemical localization of EGF, TGF- α and EGF-R in the canine testis

Fig. 2 shows representative photomicrographs of HE and immunohistochemical staining of EGF, TGF- α and EGF-R in the testes of one-year-old or older dogs. HE staining showed developed seminiferous tubules. For EGF staining, clear immunoreactivity was found in nearly all Leydig cells, and weak homogeneous staining was seen in Sertoli cells and the seminiferous epithelium (Fig. 2-b). TGF- α staining revealed stronger immunoreactivity in some but not many Leydig cells and weak and homogeneous staining in the cells of seminiferous tubules (Fig. 2-c). For EGF-R staining, stronger immunoreactivity was found in many Leydig cells than in the cells of seminiferous tubules (Fig. 2-d). For all the factors examined, no specific immunoreactivity for the stage of spermatogenesis was found in the seminiferous tubules, and positive staining was not found in the nuclei of the stained cells. After incubation with PBS instead of the primary antibody or anti-EGF, anti-TGF- α or anti-EGF-R antibodies that had been preincubated with excess antigenic peptides,

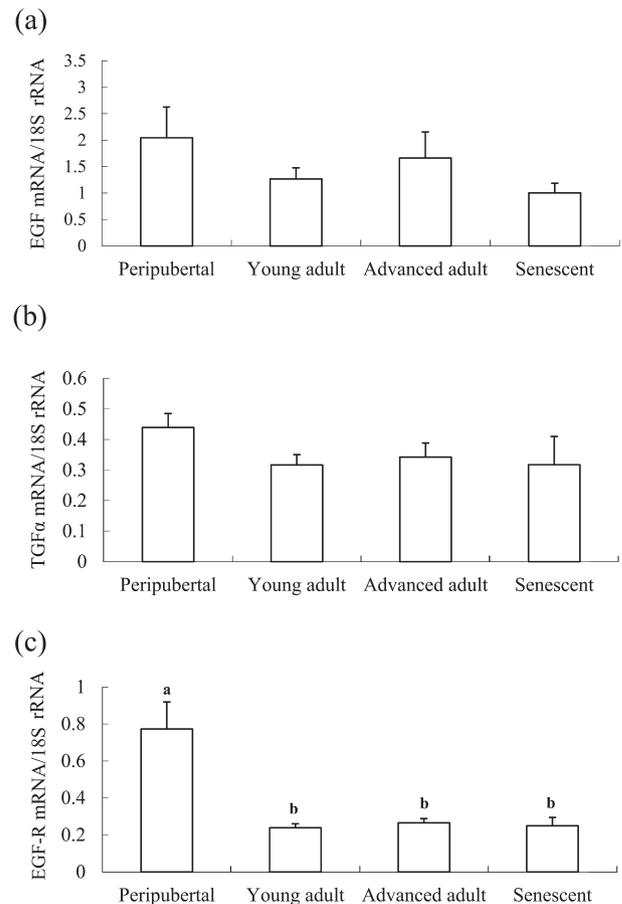


Fig. 1. Comparison of EGF (a), TGF- α (b) and EGF-R (c) mRNA levels in the canine testis among various ages. The data are presented as the mean \pm SEM. The number of animals was 5 (peripubertal, young adult and advanced adult groups) or 3 (senescent group). Values with different superscripts are significantly different between groups ($P < 0.01$).

the testicular sections displayed no staining (insets of Figs. 2-b, -c, -d). The staining patterns for EGF, TGF- α and EGF-R were not different among the breeds or ages of the dogs, whereas the intensity of the staining changed among the individual animals.

Fig. 3 shows representative photomicrographs of HE and immunohistochemical staining of EGF, TGF- α and EGF-R in the testes of 5-month-old dogs. Seminiferous tubules in 5-month-old dogs were smaller than those in the older dogs (Fig. 2-a vs. Fig. 3-a). The staining patterns for EGF, TGF- α and EGF-R were basically similar to those in the adult dog (Figs. 2-b, -c, -d). That is, stronger staining was seen in some or many Leydig cells than in the other types of cells. However, a relatively smaller number of Leydig cells with clear staining for EGF was seen in 5-month-old dogs than in adult dogs (Fig. 3-b vs. Fig. 2-b). Similar to the staining in the adult dog, some Leydig cells showed clear staining for TGF- α (Fig. 3-c vs. Fig. 2-c), and stronger EGF-R immunoreactivity was seen in many Leydig cells in the 5-month-old dogs (Fig. 3-d). The staining pattern for each factor was not different among the sections examined, whereas

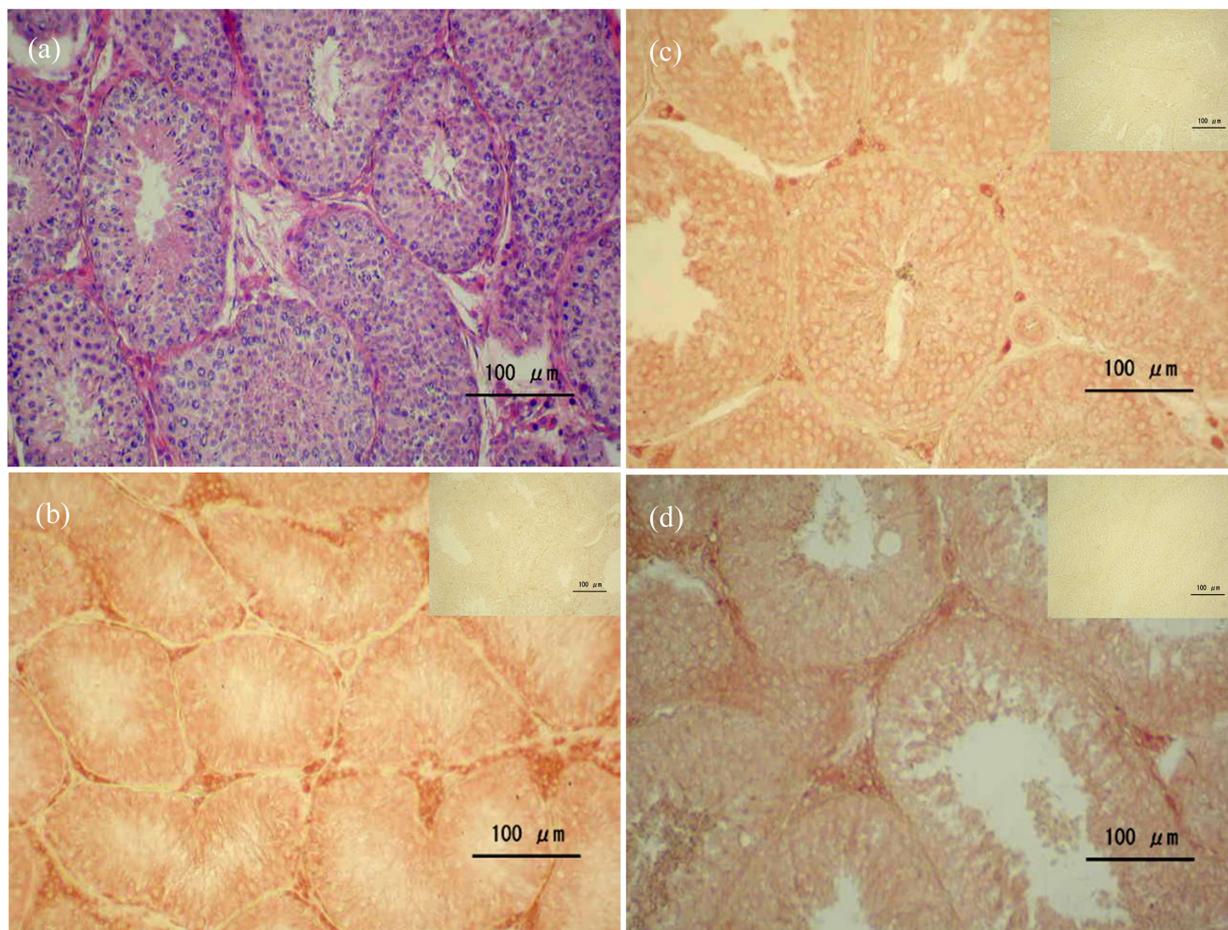


Fig. 2. Representative photomicrographs of HE (a) and immunohistochemical staining for EGF (b), TGF- α (c) and EGF-R (d) in the testes of one-year-old dogs. Negative immunostainings were observed following neutralization of the primary antibody with excess antigen (insets of each photomicrograph).

the intensity of staining changed among the individual animals.

Discussion

Spermatogenesis is regulated by not only hormones, i.e., luteinizing hormone, follicle stimulating hormone, androgens and so on, but also growth factors. Tsutsumi *et al.* [22] reported that sialoadenectomy decreased the amount of circulating EGF and inhibited the meiotic phase of spermatogenesis in mice, and administration of EGF to sialoadenectomized mice eliminated the inhibition. *In vitro* studies show that EGF stimulates steroidogenesis in Leydig cells [16] and that TGF- α facilitates proliferation of Sertoli cells [12]. While accumulating evidence indicates that the EGF family of growth factors has important roles in male reproductive functions [4, 11–18], expressions of the growth factors and their receptors have not been evaluated in the canine testis.

This study showed that the mRNAs of EGF, TGF- α and EGF-R are expressed in the canine testis and that the corresponding proteins are mainly localized in the Leydig cell. The same localization of these factors in the Leydig cell has been reported in the mouse [2,

23], rat [2] and human being [2, 9]. These findings suggest that EGF and TGF- α have some roles in the mammalian testis that function in a paracrine and/or autocrine manner.

Zhang *et al.* [24] showed that expression of the EGF gene in the mouse testis increases with maturation of the testis and proliferation of Leydig cells. In the dog, development of the seminiferous tubules is most marked between 20 and 32 weeks of age [25], and the plasma testosterone concentration increases after 22 weeks of age [26]. Consistent with this, histological sections in this study showed that the diameter of the seminiferous tubules in the peripubertal group was smaller than those in the other older groups, indicating that maturation had not begun or was occurring at that time in the testes of the peripubertal group. These findings also indicate that the ratio of Leydig cells relative to all testicular cells should be greater in peripubertal dogs than in adult dogs. If the mRNA expression levels of each type of testicular cells were not different among the groups of various ages, the levels of mRNA expression in the peripubertal dogs could be increased due to an increased ratio of Leydig cells, which may express increased mRNA for EGF, TGF- α and EGF-R compared with those of the adult dogs. However, this study showed

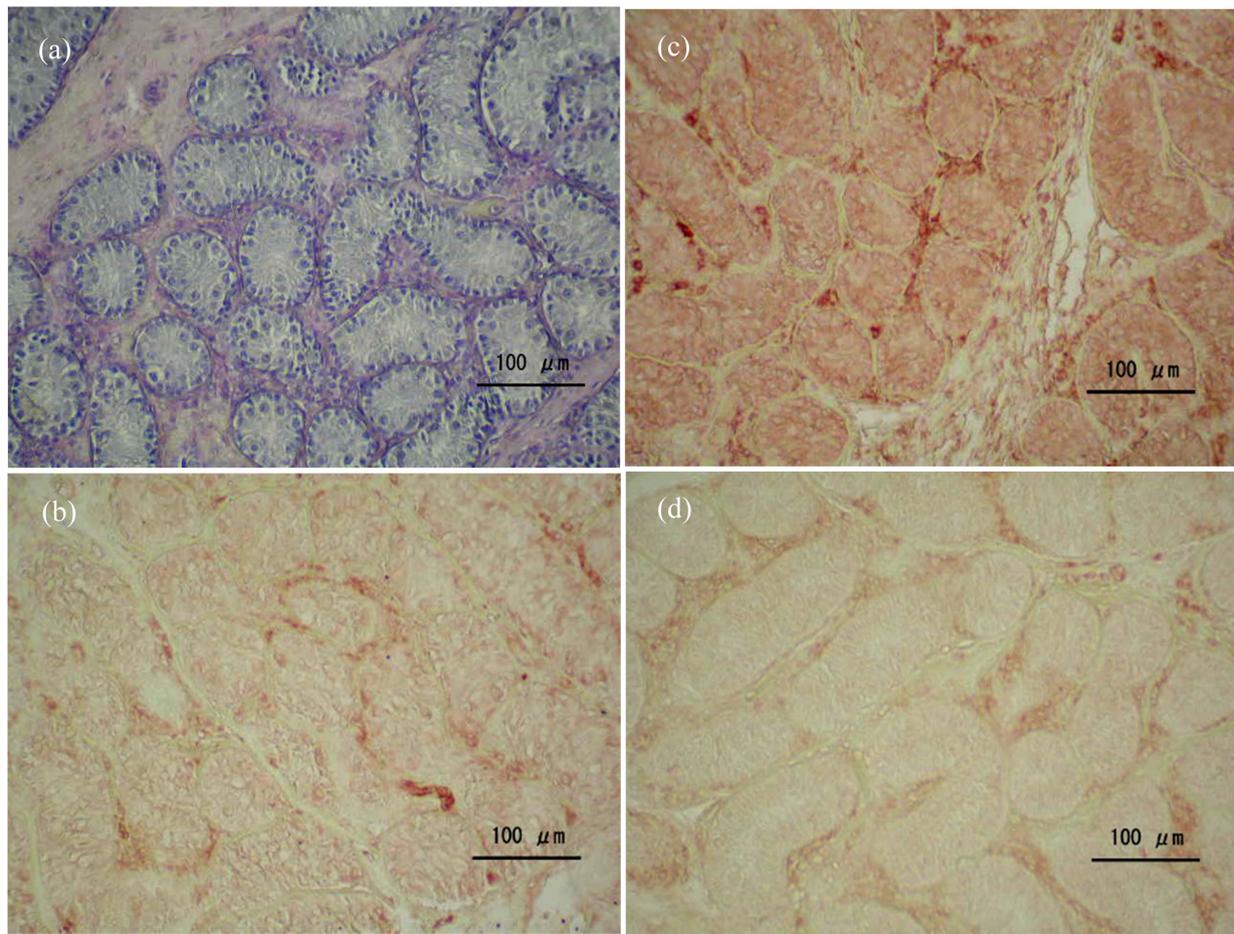


Fig. 3. Representative photomicrographs of HE (a) and immunohistochemical stainings for EGF (b), TGF- α (c) and EGF-R (d) in the testes of 5-month-old dogs.

that the mRNA level of EGF-R but not those of EGF and TGF- α was greater in the peripubertal group than in the other groups. It may be possible that the number of Leydig cells with abundant mRNA expression for EGF and TGF- α and/or their expression levels of each Leydig cells decreased in the peripubertal dog when compared to those in the adult dog. Consistent with this, immunohistochemical staining showed a smaller number of Leydig cells with clear staining for EGF in 5-month-old dogs than in adult dogs. Nonetheless, the presence of EGF, TGF- α and EGF-R mRNA and their proteins and increased levels of EGF-R mRNA in the testes of peripubertal dogs suggests that the EGF family of growth factors may be involved in testicular maturation in the dog. On the other hand, the diameter of the seminiferous tubules, spermatogenesis and the testosterone concentration in the testicular venous blood do not show age-dependent changes after maturation [27, 28]. In this study, the mRNA levels for EGF, TGF- α and EGF-R in the testes were not different among the dogs at different ages after maturation, suggesting that EGF and TGF- α are constitutively involved in the testicular functions in the dog after maturation.

In conclusion, this study showed for the first time the expression of

EGF, TGF- α and EGF-R in the canine testis. Further study is required to elucidate how the EGF family system is involved in testicular maturation, spermatogenesis and androgen secretion in the dog.

Conflict of interest: None of the authors of this paper has any financial or personal relationship with other people or organizations that might inappropriately influence or bias its content.

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