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REVIEW

Insulin-like peptide 3 in domestic animals with normal and abnormal reproductive functions, in comparison to rodents and humans

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Abstract

Background: Insulin-like peptide 3 (INSL3) is a circulating hormone secreted from only testis and ovaries in mammals. Findings on INSL3 have been gathered from subjects with normal and abnormal reproductive statuses, especially rodents and humans. However, little to no review articles focusing on INSL3 in domestic animals exist.

Methods: The author reviewed the past and recent literature regarding the structure, expression, roles of INSL3 in the reproductive organs, and its circulation under normal and aberrant reproductive conditions in domestic animals in comparison with rodents and humans.

Main findings: As with humans and rodents, blood INSL3 concentrations rise around puberty in normal male domestic animals and are associated with testicular size. INSL3 levels are acutely upregulated by luteinizing hormone (LH), and the increase is smaller than that of testosterone in male ruminants, whereas the acute regulation of INSL3 by LH does not occur in human men. Dogs with cryptorchidism and bulls with abnormal semen have lowered INSL3 levels.

Conclusion: The findings regarding INSL3 secretions in male domestic animals with normal and aberrant reproductive functions illustrate similar or dissimilar points to humans and rodents. Data on blood INSL3 levels in normal and abnormal female domestic species are still limited and require further investigation.

KEYWORDS
circulation, domestic animals, expression, insulin-like peptide 3, reproductive organs

1 | INTRODUCTION

It is well known that gonadal hormones coordinate major reproductive functions in both male and female mammals. The main secretory sources of the gonadal hormones are testicular interstitial Leydig and seminiferous Sertoli cells or ovarian follicular theca interna and granulosa cells. Testicular Leydig and follicular theca interna cells are functionally homologous between different genders, and both cells secrete androgens, including testosterone, the production of which is stimulated by luteinizing hormone (LH). In 1958, the first direct evidence that testicular androgen is produced primarily in Leydig cells was achieved using a histochemical technique.[1] Since then, androgen secreted from Leydig cells has been considered the sole androgenic hormone. In 1993, Adham et al.[2] first found mRNA coding a peptide that was expressed in porcine testicular Leydig cells,

thus named Leydig insulin-like peptide (Ley-I-L). Ley-I-L was named also as relaxin-like factor for its biological effect,[3] and is now called insulin-like peptide/factor 3 (INSL3). INSL3 is exclusively produced in the gonads, testicular Leydig cells, ovarian follicular theca interna, and luteal cells in mammals.[4,5] INSL3 concentrations in circulation increased around puberty in men. In contrast, the levels were much lower in women.[6] In male mice, INSL3 has roles in transabdominal testicular descent, which occurs in the fetal period,[7,8] and in spermatogenesis after sexual maturation.[9] INSL3 may act to maintain normal estrus cycles and fertility in mice.[8,10]

In the current review, the author reviewed the body of literature regarding the structure, expression, and roles of INSL3 in reproductive organs. The author also reviewed literature discussing INSL3's secretion and circulation, including the assay methodologies, in mammals with normal and abnormal reproductive functions, emphasizing domestic animals rather than experimental rodents and humans.

2 | INSL3 STRUCTURE, EXPRESSION, RECEPTOR, AND ROLES

2.1 | Peptide structure

In 1993, Adham et al.[2] cloned a cDNA for INSL3 from a porcine testis cDNA library. The deduced porcine INSL3 pro-form precursor consists of 131 amino acids, including a 24-amino acid signal peptide.[2] Burkhardt et al.[11] cloned a human cDNA coding INSL3 with its deduced pro-form precursor of 131 amino acids, including a 24-amino acid signal peptide. The porcine and human genome contains a single copy of the INSL3 gene, located in Chromosome 2 and 19, respectively.[12,13] Zimmermann et al.[14] isolated a mouse gene encoding INSL3 and reported that the deduced peptide consists of 122 amino acids with relatively weak homologies to human and porcine INSL3, at 73% and 71%, respectively.

The deduced INSL3 preproform consists of a signal peptide, a B-chain, a connecting (C)-peptide, and an A-chain (Figure 1A).[2,11,14,15] Büllesbach et al.[15] purified the INSL3 peptide from bovine testis and determined its amino-acid sequence for the first time. They discovered that bovine INSL3 peptides are secreted from source cells as a B-chain and A-chain heterodimer linked by two disulfide bridges (Figure 1B), where the C-peptide has been proteolytically removed.[15] Another research group suggested that human, rat, and mouse INSL3 peptides also undergo the same processing as cattle (Figure 1B).[16] However, Minagawa et al.[17] showed in pigs that the B-chain-C-peptide-A-chain pro-form monomer of INSL3 is mainly secreted into the blood circulation (Figure 1C), indicating a possible difference in the secreted form of INSL3 peptide among species due to different peptide processing mechanisms.

Figure 2 shows an alignment of deduced amino acid sequences for INSL3 peptides consisting of B-chain and A-chain among various mammals, including some domestic animals and humans. There is seemingly a higher homology of INSL3 peptides for taxonomically closer groups. For example, cattle have a homology of 95.5% with goats and sheep, 92.5% with pigs, 88.1% with horses, 83.6% with dogs, 78.8% with humans, 65.7% with rats, and 64.2% with mice.

2.2 | Expression in gonads

Since the first report of INSL3 mRNA expression in porcine testicular Leydig cells,[2] plenty of studies have localized INSL3-producing cells in the gonads of various species. It has been reported by in situ hybridization that INSL3 mRNA is exclusively expressed at high level in the testicular Leydig cells in mice,[18] cattle,[19] deer,[20] and horses.[21] Through immunohistochemistry using specific anti-INSL3 polyclonal antibodies, Ivell et al.[22] found that the peptide is produced exclusively in the Leydig cells in the human testis. The exclusive expression of the INSL3 peptide in testicular Leydig cells has been reported in mice,[23] stallions,[21] bulls,[24,25] boars,[26] and

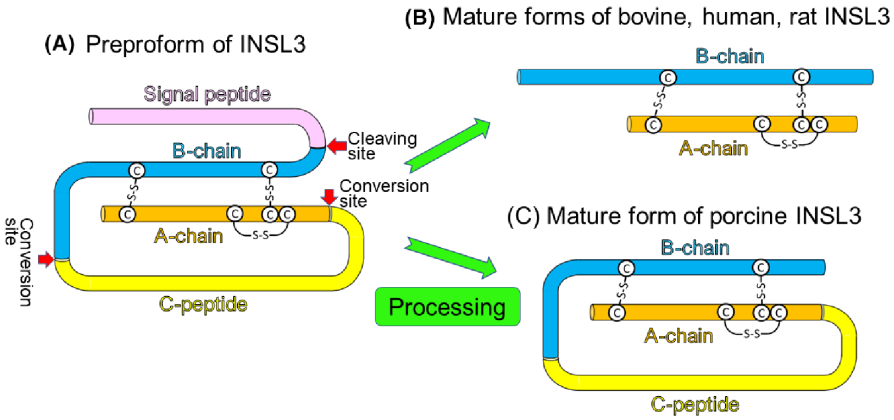

FIGURE 1 Schematic diagrams of a preproform (A), and bovine, human, rat (B), and porcine (C) mature forms of the insulin-like peptide 3 (INSL3) peptide. The preproforms of bovine, rat and human INSL3 undergo processing by removals of the signal peptide at the cleaving site and connecting (C)-peptide at the conversion sites, and then the B-chain and A-chain heterodimer is produced in the gonadal endocrine cells (B).[15,16] The preproform of porcine INSL3 forgoes the removal of C-peptide, and the resultant B-chain-C-peptide-A-chain monomer is secreted into circulation (C).[17] The "©" and "S-S" indicate a cysteine residue and a disulfide bond, respectively.

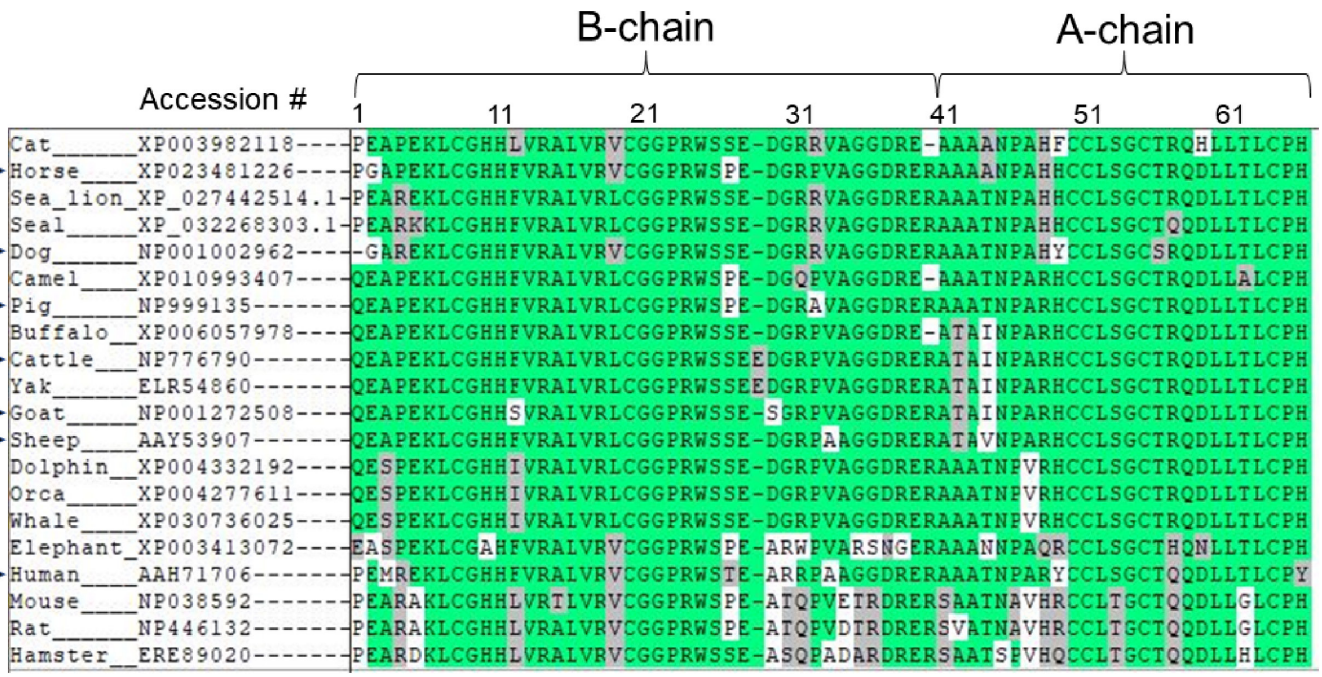

FIGURE 2 An alignment of deduced amino acid sequences for insulin-like peptide 3 (INSL3) peptides consisting of B-chain and A-chain among various mammalian species with their accession numbers of the NCBI. The matched and similar amino acids are expressed as green and gray colors, respectively. The arrowheads indicate animals whose INSL3 can be measured by the anti-bovine INSL3 mouse monoclonal antibody (2-8F) produced by Dr. Erika E. Büllesbach, Medical University of South Carolina, USA.[15]

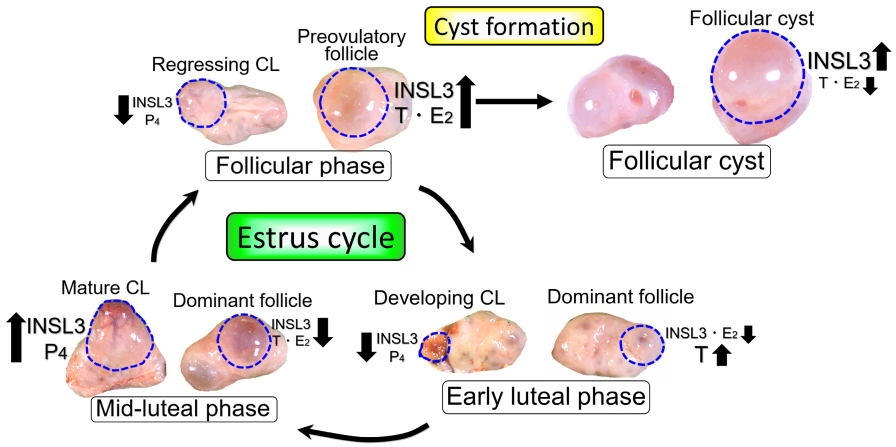

FIGURE 3 Schematic overview of INSL3 concentrations in the fluid of dominant follicles and in luteal tissues during the bovine estrus cycle, and in follicular cysts, published by Wimalarathne et al.[32] CL, corpus luteum; E_2, estradiol-17β; INSL3, insulin-like peptide 3; P_4, progesterone; T, testosterone. The upward arrows indicate higher hormonal concentrations, and the downward arrows express lower concentrations.

goats.[27] The INSL3 mRNA or peptide is also expressed in the ovarian follicular theca interna cells in cattle,[19,25,28] dogs,[29] goats,[30] and in the luteal cells in humans,[31] cattle,[19,24] mice,[23,30] goats,[30] rats,[30] and pigs.[30]

Quantitative analyses of INSL3 mRNA and peptide expressions in gonads have been reported in a few studies. Satchell et al.[25] suggested that INSL3 mRNA in bovine follicular granulosa cells increased during follicular development, measured by real-time PCR. They also showed a decline of INSL3 mRNA from the mid-luteal to regressing phase in bovine corpus luteum.[25] Wimalarathne et al.[32] revealed that INSL3 peptide concentrations in the fluid of dominant follicles of heifers are highest in the follicular phase of the estrus cycle, demonstrating that its secretion enhances bovine follicular maturation (Figure 3). Furthermore, they suggested that bovine corpus luteum produces more INSL3 peptides when it is fully matured (Figure 3).[32]

Hannan et al.[33] revealed that the total amount of INSL3 mRNA per testis in dogs declined from the pubertal age (6–12 months) to middle age (5–10 years), whereas the total amount of peptide did not change during the same period. They speculated that the change in the total amount of mRNA, but not of peptide, likely corresponds to changes in INSL3 concentrations in peripheral blood, i.e., it is highest in the pubertal age and lower in middle age.[33,34] Hannan et al. also showed reductions in the total amounts of INSL3 mRNA and peptide in the retained testis of cryptorchid dogs compared to those in normal testis.[33] These results suggest that lower blood INSL3 concentrations in bilateral cryptorchid dogs are probably due to the lower total amounts of INSL3 mRNA and peptide per retained testis.[34]

Balogh et al.[35] reported lower INSL3 mRNA concentrations in the testes of dogs treated by the sustained release of deslorelin—a gonadotropin-releasing hormone (GnRH) agonist implant—than in untreated male dogs. This finding demonstrates the downregulation of INSL3 gene expression by suppressing LH secretion. Findings also indicated higher INSL3 mRNA levels in prepubertal male dogs aged 2 months than in sexually mature dogs aged 3–4 years.[35,36] The authors inferred that the higher testicular INSL3 gene expression in prepubertal dogs might be due to a higher ratio of interstitial space, including Leydig cells, to seminiferous tubules in immature testis compared to fully-mature testis, where complete spermatogenesis takes place.[35] Further studies utilizing dissection technologies of targeted cells from tissues such as a laser capture microdissection are required to quantitate INSL3 mRNA and peptide accurately at cellular levels.[37–39]

Ferlin et al.[40] have suggested a modulating effect of INSL3 on bone metabolism and linking gene mutations of INSL3 receptors with human osteoporosis. They also demonstrated the role of INSL3 and its receptor system in protein turnover, contributing to muscle wasting in male hypogonadism.[41] Details for the roles of INSL3 in the muscolo-skeletal system are reviewed elsewhere.[42,43]

2.3 | Receptor

Büllesbach et al. synthesized human INSL3 (RLF) according to the amino acid sequence deduced from the cDNA,[11] and identified its specific binding activities to the uterus and brain in female mice.[3,44] Also, they have shown that the synthesized INSL3 has additional widening effects on pubic symphysis like relaxin.[3] Subsequently, a new gene encoding G-protein-coupled receptor affecting testicular descent (GREAT; also called LGR8 or later RXFP2) has been identified in mice by a transgene insertional mutation, which caused high intra-abdominal cryptorchidism in homozygous males.[45] Gorlov et al.[46] cloned the human GREAT gene and screened genomic DNAs of cryptorchid patients for gene mutations and identified some missense mutations in the LGR8 or RXFP2. One of the mutants for the RXFP2, T222P, in which the amino-acid substitution occurs in the extracellular domain, failed to respond in cAMP production to ligand stimulation.[46] It has been suggested that RXFP2 is the only receptor for INSL3.[47–50] Bogatcheva et al.[51,52] have demonstrated that the T222P mutation of RXFP2 is exclusively associated with human cryptorchidism. Severely reduced activity of the T222P mutant of RXFP2 is caused by the poor membrane presentation of the mutated receptor rather than impaired signal transduction.[52,53]

RXFP2 mRNA has been detected in various tissues in mice, including the gubernaculum, testis, epididymis, seminal vesicle, prostate, uterus, ovary, bladder, kidney, intestine, and brain.[46,54] Feng et al.[55] demonstrated by immunohistochemistry that the RXFP2 protein is expressed in various organs in male mice, including the gubernaculum, testis, epididymis, and kidney. In mouse testes, RXFP2 immunostaining was observed in Leydig and meiotic cells, especially in post-meiotic germ cells and, in the epididymis, an expression of RXFP2 was confined to the columnar epithelium.[55] In male goats,

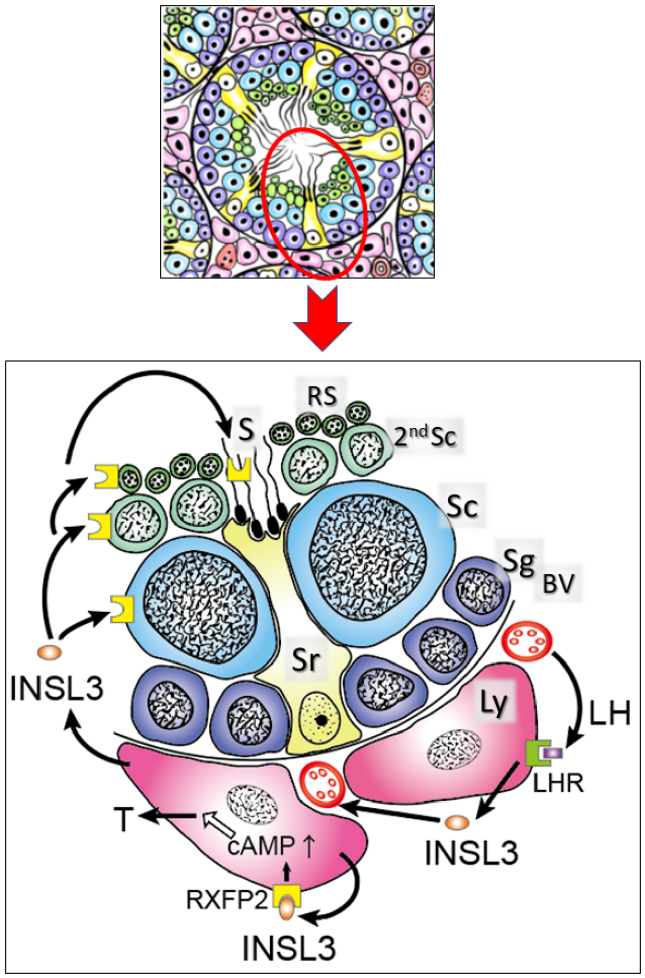

FIGURE 4 Schematic illustrations of RXFP2 localization and the roles of INSL3 in testicular tissue.[9,27,55,56,63–67] BV, blood vessel; INSL3, insulin-like peptide 3; LHR, luteinizing hormone receptor; Ly, Leydig cell; RS, round spermatid; S, spermatozoon; Sc, spermatocyte; Sg, spermatogonia; Sr, Sertoli cell; T, testosterone

RXFP2 mRNA and protein were localized to Leydig cells, meiotic and post-meiotic germ cells, the epithelium, and the smooth muscle of the cauda epididymis and vas deferens.[27] RXFP2 immunoreactivities were shown in testicular Leydig cells, seminiferous germ cells, and spermatozoa in bulls, rams, and goats (Figure 4).[56] In bovine female reproductive tissues, RXFP2 mRNA was detected in ovarian follicular theca cells, oocyte corpus luteum, and uterine myometrial cells.[25,57,58] In goats, RXFP2 was expressed in (1) luteal cells of corpus luteum, (2) follicular theca cells of ovaries, (3) tubuloalveolar and ductal epithelium of mammary glands, (4) luminal and glandular epithelium, stroma cells, and smooth muscle layer of the uterus and cervix, and (5) chorionic epithelium and syncytium of the placenta.[30]

2.4 | Roles

Zimmermann et al.[7] discovered that INSL3 plays an essential role in the testicular descent through androgen-independent

gubernaculum development, showing bilateral cryptorchidism and infertility in INSL3 gene knockout mice. Nef et al.[8] confirmed similar bilateral cryptorchidism in the INSL3-deficient male mice. Emmen et al.[59] revealed that both INSL3 and androgen are required for the outgrowth of fetal rat gubernaculum in vitro. Boockfor et al.[60] showed that INSL3 (RLF) receptors are expressed predominantly in the gubernaculum of rats; the highest level was observed a few days before parturition. They also found a growth-promoting activity of INSL3 for gubernaculum cells in vitro.[60] Hadziselimovic et al.[61] presented data showing defective development of the epididymis in the INSL3-deficient male mice, in addition to the high intraabdominal undescended position of the testes–epididymises unit. Interestingly, transgenic female mice overexpressing INSL3 displayed descent of ovaries into the inguinal hernia via the lengthening of the cranial suspensory ligament and gubernaculum.[62] Thus, INSL3 seems to have a vital role in the testicular descent through the gubernacular development in rodents. However, the role of testicular descent remains obscure in farm and companion animals.

Kawamura et al.[9] have shown that INSL3 suppresses apoptosis in testicular germ cells of adult male rats pretreated with a GnRH antagonist, demonstrating the paracrine roles of INSL3 in testicular spermatogenesis induced by gonadotropin. Administration of INSL3 antagonist into testes of sexually mature rats resulted in a decrease of the testicular weight by 20%.[63] Pathirana et al.[64] have found an autocrine role of INSL3 to stimulate testosterone secretion from cultured testicular Leydig cells through the cAMP pathway in mice (Figure 4). Sagata et al.[65] revealed that neutralizing INSL3 with long-term active immunization starting from the prepuberty increased testicular germ cell apoptosis and reduced normal sperm output in boars, suggesting that INSL3 acts as an anti-apoptotic factor in sperm production (Figure 4). The INSL3 roles of anti-apoptotic effects for spermatogenesis were confirmed by passive immunization for boars.[66] The addition of INSL3 to human sperm reportedly reduced oxidative stress and enhanced their motility.[67] The abovementioned studies advocate considerable functions of INSL3 in testicular spermatogenesis and steroidogenesis in animals, but not for humans. Direct effects of INSL3 on sperm functions remain to be determined in every species in spite of the receptor's expression on sperm in some animals. Furthermore, its tasks in male accessory reproductive organs, including seminal vesicles, prostate, and bulbourethral glands, which affect semen characteristics, are totally unknown.

In females, it has been found that the INSL3-deficient mice have a prolonged estrus cycle and impaired fertility.[8] Spanel-Borowski et al.[10] have shown that follicular atresia and luteal-cellular apoptosis are accelerated in ovaries of INSL3-deficient mice, suggesting a function of INSL3 to rescue endocrine cells from the apoptotic pathway. Kawamura et al.[9] displayed that treatments with INSL3 initiate meiotic progression of arrested oocytes in preovulatory follicles of rats in vitro and in vivo. There have been a couple of studies showing the effects of INSL3 in steroidogenesis of bovine ovarian endocrine cells. Glister et al.[68] showed up-regulation of androstenedione secretion by INSL3 in primary cultures of theca interna cells from bovine small antral follicles, suggesting its autocrine roles for ovarian follicular androgen production. Dai et al. found that a low level of LH and growing follicular levels of estrogen enhanced INSL3 production using the same primary cell cultures of bovine theca interna, as has been shown by Glister et al.,[68] postulating a feedforward loop driving INSL3 secretion, which leads to higher estrogen production in the growing antral follicle.[69] Using a primary cell culture from bovine corpus luteum, Abe et al.[57] revealed that INSL3 stimulated progesterone secretion from luteal cells. Li et al.[70] have shown that reduced INSL3/RXFP2 signaling on Caveolin-1-deficient mice caused the development of endometrial cysts, indicating functional significance of INSL3 in the uterus. Further studies are necessary to elucidate the roles of INSL3 in oogenesis and reproductive tracts, including the oviduct and uterus, in domestic animals and humans.

3 | ASSAYS TO MEASURE CIRCULATING INSL3

Various assay methods have been developed in several laboratories to measure INSL3 concentrations in the peripheral blood of mammals (Table 1). In 1999, Büllesbach et al.[6] undertook the first reported instance with a homogeneous competitive radioimmunoassay using human INSL3 as the standard and antibodies raised against the human INSL3 to measure serum concentrations in men. Following the first report, competitive time-resolved fluorescence immunoassays (TRFIA), competitive enzyme immunoassays (EIA) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) were reported to measure blood INSL3 in humans,[71,72] rats,[60,73] cattle,[74–76] sheep,[74] dogs,[34] pigs,[26] goats,[77,78] and horses[79] (Table 1). The author's research group has been using the anti-bovine INSL3 mouse monoclonal antibody (2-8F; produced by Dr. Erika E. Büllesbach, Medical University of South Carolina, USA),[15] which can be used to measure blood INSL3 peptides in multiple species, including cattle,[75,76,80] goats,[77,78,81] sheep,[82] pigs (unpublished data), horses,[79,83] dogs,[34] and humans (unpublished data) (Figure 2). It should be noted that all the immunoassays were performed with a single anti-INSL3 antibody; thus, only the competitive assay has been used until now. To improve the specificity and sensitivity of the immunoassay, an advent of sandwich assays would be anticipated, in which two types of antibodies recognizing different epitopes of the peptide are essential. The detection limit of LC–MS/MS, which requires pretreatment including reduction, alkylation, and evaporation steps, was 0.06 ng/ml and equivalent to RIA or TRFIAs.

4 | INSL3 SECRETIONS IN NORMAL ANIMALS AND HUMANS

4.1 | Circulating INSL3 in males with normal reproductive functions

Büllesbach et al.[6] reported for the first time that serum INSL3 concentrations increased from prepuberty to post-puberty in men; in women, the concentrations were much lower than in men. In the second trimester of human pregnancy, INSL3 concentrations in the

TABLE 1 Assays to measure blood INSL3 in mammals and their characteristics

Year published	Species	Samples	Methods (tracer, homo- or hetero-logous antibody)	Detection limit	Range	Authors and references
1999	Human	Serum	Competitive RIA (^{125}I, homo)	0.06 ng/ml	0.06–6 ng/ml	Büllesbach et al.[6]
2001	Rat	Serum	Competitive RIA (^{125}I, hetero)	0.1 ng/ml	0.1–25 ng/ml	Boockfor et al.[60]
2005	Human	Serum	Competitive TRFIA (europium, homo)	0.05 ng/ml	0.05–3.2 ng/ml	Bay et al.[71]
2009	Rat	Plasma	Competitive TRFIA (europium, homo)	0.02 ng/ml	0.02–5 ng/ml	Anand-Ivell et al.[73]
2011	Cattle, Sheep	Serum	Competitive TRFIA (europium, homo for cattle)	0.02 ng/ml	0.02–20 ng/ml	Anand-Ivell et al.[74]
2011	Cattle (Bull)	Plasma	Competitive EIA (HRP, homo) with extraction	0.5 ng/ml	0.5–20 ng/ml	Kawate et al.[75]
2012	Dog	Plasma	Competitive TRFIA (europium, hetero)	0.02 ng/ml	0.02–20 ng/ml	Pathirana et al.[34]
2014	Pig	Serum	Competitive TRFIA (europium, homo)	0.16 ng/ml	0.16–160 ng/ml	Minagawa et al.[26]
2016	Goat	Plasma	Competitive EIA (HRP, hetero)	0.3 ng/ml	0.3–20 ng/ml	Hannan et al.[77]
2017	Goat	Plasma	Competitive TRFIA (europium, hetero)	0.07 ng/ml	0.07–20 ng/ml	Hannan et al.[78]
2018	Cattle (Bull)	Plasma	Competitive TRFIA (europium, homo)	0.15 ng/ml	0.15–20 ng/ml	Weerakoon et al.[76]
2018	Human	Serum	LC–MS/MS with pretreatment	0.06 ng/ml	0.15–5 ng/ml	Albrethsen et al.[72]
2019	Horse	Plasma	Competitive TRFIA (europium, hetero)	0.15 ng/ml	0.15–20 ng/ml	Hannan et al.[79]

Abbreviations: EIA, enzyme immunoassay; HRP, horseradish peroxidase; LC–MS/MS, liquid chromatography–tandem mass spectrometry; RIA, radioimmunoassay; TRFIA, time-resolved fluorescence immunoassay.

amniotic fluid of male fetuses were higher than those of females, where the INSL3 was undetectable, suggesting that INSL3 could be involved in the abdominal testis translocation in humans.[84] In male infants, serum INSL3 concentrations were high for a few months after birth, decreased sharply to nadir levels between a few months and 1 year of age,[85] remained low until 10 years of age, and thereafter increased during puberty; in contrast, INSL3 was unmeasurable in girls at all ages.[86,87] In rats, serum INSL3 concentrations decreased rapidly from 2 days before birth to 3 days after birth, remained low up to 10 days after birth, and then increased to pubertal age.[60,73] The authors presented changes in plasma INSL3 levels in bulls for the first time from birth through post-puberty; the concentrations continuously increased during the first 3 months after birth, followed by no changes from prepuberty to early puberty and then another rise from late puberty to post-puberty (Figure 5A).[75,80] Minagawa et al.[26] reported similar increments of serum INSL3 concentrations from prepuberty to post-puberty in boars. The authors also suggested increases in plasma INSL3 concentrations from early puberty to late and post-puberty in male goats (Figure 5B).[81] In male dogs, the authors observed a temporal rise of INSL3 levels from prepuberty to puberty, followed by a decline in post-puberty.[34] Plasma INSL3 concentrations in male horses rose from birth to prepuberty but did not change from prepuberty to early puberty.[79] Thus, the blood INSL3 concentrations rise around puberty in male domestic animals as well as humans and rodents. However, decreased INSL3 secretion between the neonatal and pubertal periods observed for men and male rats is absent in bulls.[75,80] The higher level of INSL3 observed around the neonatal period in rats and humans might be associated with the final stage of testicular descent into the scrotum, which occurs around this period.[88] In contrast to those species, bovine testicular descent completes around 4 months of fetal age,[88] during mid-pregnancy in cattle, and thus such an increase of INSL3 secretion around the birth may be unnecessary in bulls.

In most of the abovementioned studies, INSL3 and testosterone concentrations—both secreted from Leydig cells—were measured in the same blood samples.[26,34,75,78–81,86] Those studies showed different changes between INSL3 and testosterone concentrations around puberty (Figure 5), suggesting a differing mechanism for both hormonal secretions. The authors have shown by serial blood sampling with 15-min intervals for 8 h that pulsatile INSL3 secretion into blood circulation was acutely upregulated by LH pulses and that the increase of INSL3 by the LH was much lower than that of testosterone in bulls and male goats (Figure 6).[77,89] Also, we have suggested that suppression of pulsatile LH release by a long-acting GnRH antagonist, degarelix acetate, induced immediate reduction of testosterone secretions followed by INSL3 with a few days delay in male goats, indicating a slower decrease of INSL3 secretion in response to reductions of pulsatile LH release than that of testosterone.[78,90] Additions of human chorionic gonadotropin (hCG), which has LH actions, to cultured canine testicular interstitial cells for

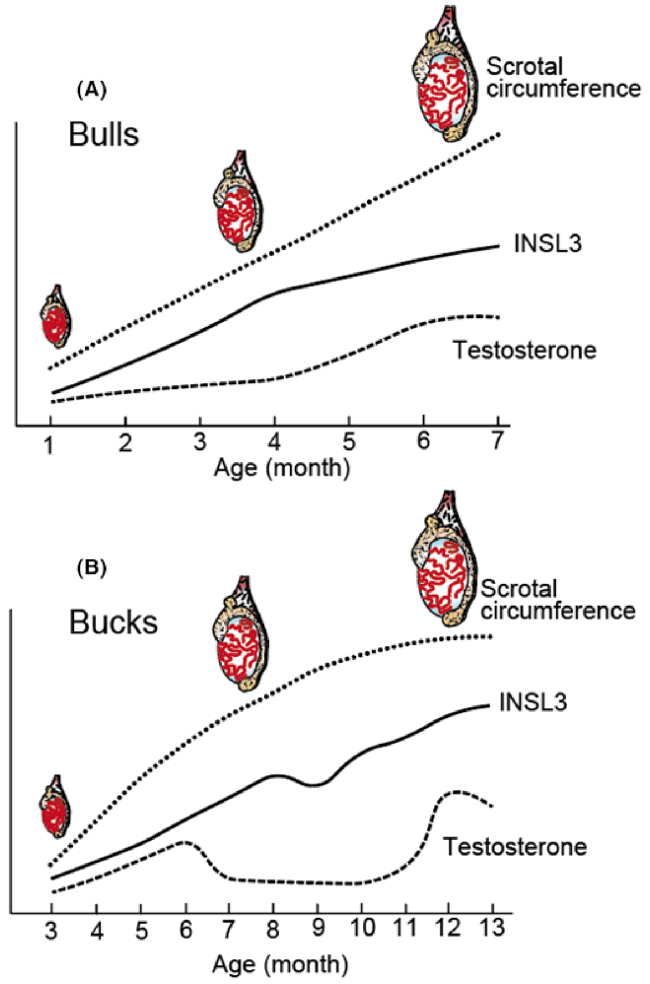

FIGURE 5 Schematic diagrams of changes in blood insulin-like peptide 3 (INSL3) concentrations and scrotal circumference from the neonatal period to the prepuberty in bulls (A)[75,80] and around the puberty in male goats (bucks) (B)[81]

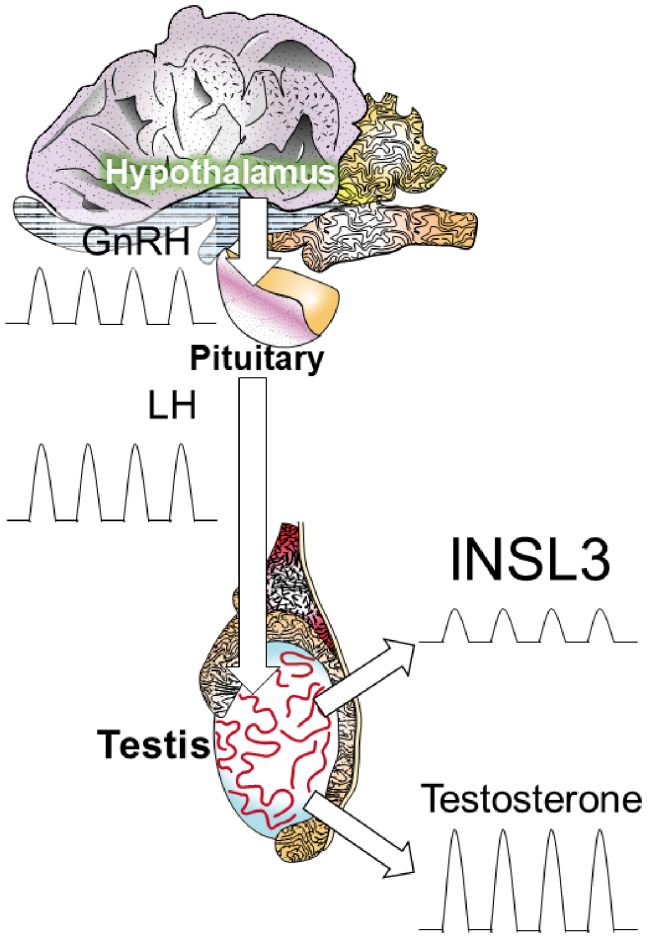

FIGURE 6 Schematic diagrams of pulsatile release of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), insulin-like peptide 3 (INSL3), and testosterone in the circulation of bulls.[89] and bucks[77,78]

18 h stimulated INSL3 release in vitro.[91] On the other hand, in men, serum INSL3 levels did not change by an hCG treatment in a daily blood sample for 8 days, whereas testosterone increased for a few days after treatment,[92] suggesting that the INSL3 secretion is not acutely regulated by LH.[93]

The authors found that scrotal circumference, which is a good indicator of testicular volume, is correlated more highly with plasma INSL3 concentrations than with testosterone during pubertal development of male goats (Figure 5B).[81] We also suggested that blood INSL3 concentrations may be a better functional indicator than other testicular hormones, such as testosterone and inhibin, for determining total testicular volume during prepuberty in bull calves (Figure 5A).[80] In men, it has been illustrated in multiple articles that serum INSL3 levels represent a potent biomarker of testicular Leydig cell differentiation and function.[86,92,94,95]

In a large population of Australian men, serum INSL3 concentrations declined clearly from a group of subjects aged 35–44 years to one featuring those aged 75–80 years.[96] However, the effects of aging on circulating INSL3 concentrations are unknown in domestic animals.

4.2 | Circulating INSL3 in females with normal reproductive functions

Due to lower INSL3 levels in blood circulation, limited information is available on female animals. Satchell et al.[25] showed a slight rise in plasma INSL3 concentration on the next day of prostaglandin $F_{2\alpha}$ treatment, followed by a decline on the fourth day in dairy heifers. In healthy young women, circulating INSL3 concentrations rose from menses to the early follicular phase and reduced from the luteal phase to menses.[97] Dai et al.[69] reported that INSL3 secretion was stimulated by LH in cultured bovine theca interna cells. Concentrations of INSL3 mRNA in bovine luteal tissues did not change from the early luteal to mid-luteal phase but decreased from the mid-luteal to regressed phase;[25] those of RXFP2 mRNA were highest at the early luteal phase and decreased toward the regressed phase.[57]

It has been reported that blood INSL3 concentrations at mid or late gestation in cattle carrying male fetuses were higher than those carrying females.[74,98] One research group suggested the feasibility of predicting fetal gender by utilizing higher maternal INSL3 and testosterone concentrations at mid and late gestation in dairy and beef cattle.[98] More studies are required to understand the changes and regulations of circulating levels and the possible roles of INSL3 in female reproduction of domestic animals.

5 | INSL3 SECRETIONS IN REPRODUCTIVE DISORDERS

5.1 | INSL3 secretions in males with abnormal reproductive functions

Bay et al.[71] reported that serum INSL3 levels in anorchid men were below the detection limit (0.05 ng/ml), whereas the average of INSL3 concentrations is 0.99 ng/ml (range: 0.55–1.73) in normal adult men. In adult men with Klinefelter's syndrome, which is a chromosomal disorder causing smaller testis and azoospermia, serum INSL3 concentrations were lower than in normal adult men.[99,100] Ferlin et al.[99] also showed lower INSL3 levels in infertile men with severe hypospermatogenesis than normal adult men, but higher than patients with Klinefelter's syndrome. Serum INSL3 levels in the cord blood of cryptorchid Finnish boys were lower at birth than those of the normal control group, but the levels in peripheral blood at 3 months of age did not differ between cryptorchid and normal boys.[87] In this article, they also showed a higher ratio of LH per INSL3 levels in peripheral blood in cryptorchid boys at 3 months, suggesting the occurrence of a mild degree of Leydig cell dysfunction already during the perinatal period in human cryptorchidism.[87] Emmen et al.[101] revealed that treatment with diethylstilbestrol for pregnant mice inhibited transabdominal descent of testis in male fetuses, and at the same time, INSL3 mRNA was reduced in the gubernaculum, proposing a possible mechanism of cryptorchidism by lowered INSL3 secretion.

In male small-breed dogs, Pathirana et al.[102] demonstrated that plasma INSL3 concentrations, as well as testosterone, were lower in bilateral cryptorchid than in normal and unilateral cryptorchid animals. In an in vitro study using canine testicular interstitial cells, LH-induced secretory testosterone and INSL3 responses were lower in retained testes than in scrotal testes; and that high concentrations of LH may acutely stimulate INSL3 release in scrotal testes of dogs but not in retained testes.[91,102] Hannan et al.[33] suggested that INSL3 in retained testes of cryptorchid small-breed dogs is substantially expressed per unit-weight basis but may be produced with a lower amount as a whole testis. Also, their study provided findings that the RXFP2 gene is barely expressed in the retained testes but normally in cryptorchid scrotal testes.[33]

Weerakoon et al.[76] suggested reduced INSL3 concentrations in blood plasma surrounding puberty may be associated with semen aberration, especially morphological abnormality and low motility of sperm in fresh semen, in Japanese Black beef bulls. On the other hand, plasma LH and testosterone concentrations after a GnRH challenge did not differ between the beef bulls with normal and abnormal semen at 20 months of age.[103] In pubertal dairy bulls, which were experimentally fed with a low plane of nutrition from calfhood to 6 months of age, serum INSL3 concentrations as well as body weights, scrotal circumferences, and sperm concentrations were lower and age at when they reached puberty was also higher in bulls given a high plane of nutrition.[104]

5.2 | INSL3 secretions in females with abnormal reproductive functions

Articles regarding circulating INSL3 in female reproductive disorders are limited to a few publications about polycystic ovarian syndrome (PCOS) and a single article on premature ovarian insufficiency (POI) in women. Gambineri et al.[105] showed for the first time that serum INSL3 levels in women with PCOS are related to LH and ovarian androgenic function, suggesting that INSL3 may be considered a circulating hormone related to LH-dependent ovarian hyperandrogenism, especially in normal-weight (BMI of $<25\,kg/m^2$) PCOS women, not in overweight (BMI of $\geq 25\,kg/m^2$) patients. Szydlarska et al.[106] also found a positive correlation between INSL3 and androgens in PCOS women, especially those with normal weights. Gambineri et al.[107] also reported in PCOS women—most of which were overweight—that INSL3 is related to exaggerated 17-hydroxyprogesterone responses to GnRH, and thus INSL3 is related to the functional ovarian hyperandrogenism in PCOS women. However, this association is not likely mediated by LH.[107] Pelusi et al.[108] examined circulating levels of anti-müllerian hormone (AMH), which is secreted from follicular granulosa cells, in addition to INSL3, in PCOS women categorized by menstrual status into eumenorrheic, oligomenorrheic, and amenorrheic groups. They found that INSL3 and AMH levels are positively correlated in women with PCOS, and both hormones are increased, particularly in amenorrhea and oligomenorrhea, suggesting both hormones reflect a dysfunction of PCOS thecal and granulosa cells.[108] Further studies are required to determine whether the circulating INSL3 levels would be a potential marker for the diagnosis of PCOS in women.[109]

Recently Zhu et al.[110] reported that serum INSL3 concentrations in women with POI, also called primary ovarian insufficiency, are defined by the cessation of ovarian function before the age of 40 years, reduced compared to age-matched control women. Antral follicular number in a pair of ovaries of the POI patients examined by ultrasonography was zero, whereas that of the control normal women was 13.[110]

Wimalarathne et al.[32] have presented that INSL3 concentrations in the fluid of bovine follicular cysts are comparable to those of dominant follicles at the follicular phase, whereas testosterone levels are lower in the cysts, suggesting that bovine follicular cysts may retain a capacity to secrete INSL3 during the formation (Figure 3). However, in female domestic animals with ovarian diseases, information on

circulating INSL3 concentrations is currently lacking, and thus the relevant studies would be highly welcomed.

6 | CONCLUSION

Bovine, human, and rat INSL3 peptides are secreted as B-chain and A-chain heterodimers linked by two disulfide bridges, whereas the porcine version circulates as a monomer without proteolytical cleavage of the connecting peptide between both chains. Among some mammals, an alignment of deduced amino acid sequences of the heterodimer indicates higher homology of the peptides for taxonomically closer species. The production site of INSL3 is limited to gonadal endocrine cells, Leydig, and theca interna cells in various animals. The first discovered role of INSL3 in mice is to induce a testicular descent through promoting gubernacular growth. The INSL3 receptor, RXFP2, is expressed in the fetal gubernaculum and in a wide variety of organs after puberty, not only in gonads and reproductive tracts but also in extra-gonadal organs. INSL3 is produced in the gonadal endocrine cells, probably at higher levels after puberty when the germ cells functionally mature since the peptide has roles in gonadal spermatogenesis and oogenesis and steroidogenesis in several species.

Several assay methodologies such as competitive RIA, TRFIA, EIA, and LC–MS/MS have been developed to quantify INSL3 concentrations in blood and body fluids for some domestic animals, humans, and rodents. Serum INSL3 concentrations increase from prepuberty to post-puberty in men; the concentrations are much lower in women. Blood INSL3 concentrations rise around puberty in normal male domestic animals, as is the case in humans and rodents, implying the peptides' possible role in the onset of spermatogenesis in domestic animals. INSL3 secretion into blood circulation is acutely upregulated by LH, and the increase of INSL3 by the LH is much lower than that of testosterone in bulls and bucks, whereas such an acute regulation by LH is unlikely to occur in human males. The blood INSL3 concentration can be a good marker for testicular size in bulls and bucks, as with human men. In normal female animals and humans, limited information is available regarding the INSL3 level in blood circulation because of its lower concentrations. Men with anorchidism and Klinefelter's syndrome, and cryptorchid boys show reduced blood INSL3, suggesting that the circulating peptide concentrations are a biomarker for the malfunction of Leydig cells. Bilateral cryptorchid dogs and bulls with semen aberration also have lowered plasma INSL3 levels. Some women with polycystic ovarian syndrome show higher INSL3 levels, whereas patients with premature ovarian insufficiency have decreased concentrations. Information on circulating INSL3 in female domestic animals is limited, and further investigation is required.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

HUMAN AND ANIMAL RIGHT

The review article does not contain any studies with human subjects conducted by the author. No animal experiments were carried out in the review article.

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