称号及び氏名	博士(獣医学)　Wimalarathne Hewage Dilhan Anuradha
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論　文　名	Secretion of insulin-like peptide 3 in female cattle and expression of its receptor in oocyte and sperm (雌ウシにおけるインスリン様ペプチド 3 の分泌および卵母細胞と精子における同受容体の発現)
論文審査委員	主査　川手 憲俊 副査　桑村 充 副査　山岸 則夫 副査　鳩谷 晋吾

論文要旨

Introduction

Insulin-like peptide 3 (INSL3) is produced exclusively by testicular Leydig cells in males and by theca interna cells of ovarian antral follicles and luteal cells in females. INSL3 is considered a hormone owing to its ability to circulate in the peripheral blood in male animals including bulls. However, information regarding circulating INSL3 levels in female cattle is very limited due to its lower concentrations. Hence, introducing sandwich immunoassays, utilizing two antibodies for enhanced sensitivity and specificity, is crucial for accurately quantifying peripheral INSL3 levels in female cattle. No previous studies have examined changes in circulating INSL3 levels during growth from calves to cows, across different reproductive conditions, including follicular and luteal phases, and in the presence of ovarian disorders in cows. Moreover, INSL3 secretions in bovine dominant follicles and corpus luteum throughout the estrus cycle have not yet been investigated.

It has been suggested that biological actions of INSL3 in target cells are mediated through relaxin family peptide receptor 2 (RXFP2) located on the cell surface. RXFP2 mRNA was detected in bovine ovarian follicular theca cells, oocytes and corpus luteum. Until now, there have been no reports to quantitate the RXFP2 protein in bovine oocytes of antral follicles in differing sizes. The RXFP2 protein was previously detected in bovine spermatozoa from frozen semen. However, quantitative comparison of RXFP2 protein in bovine sperm between normal and abnormal semen has not been carried out.

In Chapter 1 of this study, changes of circulating INSL3 concentrations were determined in female Japanese Black beef cattle across different age groups and reproductive conditions with a newly developed sandwich time-resolved fluorescence immunoassay. In Chapter 2, INSL3 was quantified in dominant follicles and corpora lutea during the estrus cycle and in the follicular cysts in beef heifers. Then, RXFP2 expressions were analyzed in bovine oocytes of antral follicles and in spermatozoa in normal and abnormal semen of beef bulls in Chapter 3.

Chapter 1: Circulating INSL3 concentration in female beef cattle: changes with age and their associations with ovarian conditions

The objectives of the present study were to 1) develop a sandwich time-resolved fluorescence immunoassay (TRFIA) with higher detectability to measure blood INSL3 concentrations in female cattle, 2) determine INSL3 concentrations in female cattle among age groups and reproductive conditions, and 3) explore associations between INSL3 levels and ultrasonographic ovarian measurements. Blood was collected repeatedly from Japanese Black beef female calves (0–8 mo), heifers (10–26 mo) and cows (27–200 mo). Blood was taken from cows at follicular, postovulatory, and luteal phases, and from cows with follicular cysts. Ultrasonography of ovaries was conducted in the calves and the cows without ovarian diseases. The number and diameters of antral follicles ≥ 2 mm were determined in each ovary.

The new sandwich TRFIA detected a difference in plasma INSL3 between calves (0.01 ng/mL) and heifers (0.18 ng/mL). However, the conventional assay showed similar levels for calves and heifers (1.82 vs 2.07 ng/mL). Plasma INSL3 and testosterone concentrations increased from calves to heifers ($P < 0.0001$), but only INSL3 rose from heifers to cows ($P < 0.0001$). INSL3 and testosterone concentrations did not change across the estrus cycle in cows, and the levels of both hormones in follicular cystic cows did not differ from those in the follicular phase. Plasma INSL3 concentrations correlated positively with the total volumes of all follicles in calves ($P < 0.05$) and cows ($P < 0.05$), whereas testosterone concentrations did not correlate with ovarian follicular measurements.

In conclusion, plasma INSL3 concentrations measured by the proposed sandwich TRFIA showed a clear increase from female calves to cows in beef cattle. These results suggest that circulating levels of INSL3, but not of testosterone, are associated with the total volume of all antral follicles in both ovaries per animal in female cattle.

Chapter 2: Quantitative analyses of INSL3 and sex steroid hormones in dominant follicles and corpora lutea during the estrus cycle and in follicular cysts in beef heifers

In the initial chapter, our research revealed consistent plasma INSL3 concentrations across various stages of the bovine estrus cycle, including cows with follicular cysts when compared to those in the follicular phase. The consistent INSL3 levels in blood during the estrus cycle indicate a possibility that INSL3 may function as a local hormone acting on the ovary itself and nearby genital tracts in female cattle. This chapter is dedicated to measuring ovarian INSL3 secretion, focusing on the turnover of dominant follicles and follicular maturation during the bovine estrus cycle, follicular cyst formation, and luteal development and regression. Paired ovaries from beef heifers were classified, by their morphological features, either into four stages of the estrus cycle (Day 1 = day of ovulation, Day 20 = day of estrus) as Stage I (Days 1–4),

Stage II (Days 5–10), Stage III (Days 11–17), and Stage IV (Days 18–20) or follicular cystic. Cysts were subdivided into estrogen-active and estrogen-inactive cysts.

INSL3, testosterone, and estradiol-17β concentrations in the dominant follicular fluid of Stage IV were higher than those in Stages II and III ($P < 0.05$). INSL3 concentrations in the cystic fluid were like those in dominant follicles at Stage IV, whereas testosterone and estradiol-17β concentrations were lower in cysts ($P < 0.05$). INSL3 content per estrogen-inactive cyst was higher than that of Stage IV ($P < 0.05$). INSL3 and progesterone concentrations in luteal tissue and contents per corpus luteum were higher in Stages II and III ($P < 0.05$).

In conclusion, INSL3 secretion in bovine dominant follicles increased with maturation. Follicular cysts may retain the production of INSL3 during their formation but tend to lose the capacity for testosterone secretion. Estrogen-inactive cysts subjected to advanced atresia may accumulate more INSL3. INSL3 production in bovine corpora lutea is enhanced during maturation.

Chapter 3: RXFP2 protein expression in oocytes of bovine antral follicles and in spermatozoa in normal and abnormal semen of beef bulls

The Chapter 2 revealed greater INSL3 secretion in the preovulatory follicles during the estrus cycle. In the preovulatory follicles, which are fully matured antral follicles grown up from small antral follicles, INSL3 may stimulate oocytes directly to assist their maturation process during follicular development and maturation prior to ovulation. Given that preovulatory follicles eventually outpour oocytes with follicular fluid containing large amount of INSL3 into the oviduct, the hormone might affect sperm waiting for the forthcoming fertilization. In this chapter, the following studies were performed to elucidate the expression levels of RXFP2 protein in oocytes from bovine antral follicles and in sperm of fresh semen from beef bulls.

Section I: RXFP2 in bovine oocytes of antral follicles differing in size

INSL3 exerts its effects by binding to the RXFP2. The RXFP2 mRNA expression was previously detected in bovine oocytes, whereas it remains poorly studied in RXFP2 protein expression in bovine oocytes in different sizes of antral follicles. The objective of this section is to characterize the protein expression of RXFP2 in bovine oocytes derived from small (2–8 mm in diameter) and large (10–18 mm in diameter) antral follicles. Protein expression of RXFP2 of the oocytes was assessed by the immunofluorescence with confocal laser microscopy and obtained images were quantified by an image-analyses software. INSL3, testosterone, and estradiol-17β concentrations in small and large follicles were measured by either TRFIA, or EIA.

As the results, RXFP2 protein was found in the bovine oocytes, with higher total RXFP2 expression observed in oocytes from small follicles compared to those from large follicles ($P < 0.05$). Higher concentrations of INSL3 and testosterone were found in the small follicular fluid than in the large follicular fluid ($P < 0.05$), whereas estradiol-17β levels were lower in the small follicular fluid ($P < 0.05$).

These results suggest that bovine oocytes are direct target cells for INSL3. Higher INSL3 concentrations in small follicular fluid and greater expression of its receptor protein in oocytes from small follicles may imply some roles in the germ cells of the follicles.

Section II: RXFP2 in sperm and INSL3 in seminal fluid between normal and

abnormal semen of beef bulls

It has been shown that RXFP2 is expressed on head and neck parts of bovine sperm. However, an association of RXFP2 protein on bovine sperm with its morphology is unknown. Also, INSL3 levels in seminal fluid of bulls are yet to be determined. The objective of this section is to examine the relationships of RXFP2 expression in sperm and the testicular hormonal concentrations in seminal fluid with sperm morphology in fresh semen from beef bulls. Ejaculates were collected from yearling Japanese Black beef bulls. Collected semen was evaluated for its sperm morphology and categorized into three groups: High (normal morphology ≥ 80 %), Mid (<80 % & ≥ 65 %) and Low (< 65 %). Sperm was subjected to the same immunofluorescence method used for the oocytes to determine the expression levels of RXFP2. The seminal plasma composition was analyzed for INSL3 and testosterone using TRFIA and EIA respectively.

The results showed that RXFP2 protein was expressed at the acrosome, equatorial, post-acrosomal regions in the head part and at the neck part of the sperm in all groups. RXFP2 expressions at the acrosomal and post-acrosomal regions, and at the neck part in Low group were lower than those in High and Mid groups ($P < 0.05$) whereas the expression at the equatorial region was similar among all three groups. Total RXFP2 expression in the sperm of the Low groups was also reduced compared to those in High and Mid groups ($P < 0.05$). Seminal INSL3 concentrations were higher in the Low group compared to the High and Mid groups ($P < 0.05$) whereas testosterone concentrations did not show any difference among the groups. Seminal fluid INSL3 concentrations negatively correlated with the sperm morphological normality ($P < 0.05$) whereas testosterone concentrations did not.

Our findings underscore differential expression levels and distribution of RXFP2 expression in relation to sperm morphology, suggesting possible roles in sperm capacitation. Abnormal semen with more malformed sperm exhibited reduced RXFP2 expression, suggesting that the higher INSL3 levels in the seminal fluid occurred due to less INSL3 binding on sperm in the abnormal semen. Seminal INSL3 levels were negatively correlated with sperm morphological normality, whereas testosterone did not, highlighting potential values of seminal INSL3 as a biomarker to assess bulls' fertility.

Overall conclusions

1. Plasma INSL3 concentrations measured by the newly developed sandwich TRFIA showed a clear increase from female calves to cows in beef cattle.
2. Circulating levels of INSL3, but not of testosterone, were associated with the total volume of all antral follicles in both ovaries per animal in female cattle.
3. INSL3 secretion in bovine dominant follicles increased with maturation.
4. Bovine follicular cysts may retain the production of INSL3 during their formation and corpus luteum might produce more INSL3 upon full maturation.
5. INSL3 may directly act on bovine oocytes and have potential roles in the germ cells.
6. RXFP2 was reduced in the head and neck parts of bovine sperm in abnormal semen with more malformed sperm. Thus, INSL3 may have possible roles in sperm capacitation.
7. Seminal INSL3 levels might have potential values as a biomarker to assess bulls' fertility.

審査結果の要旨

哺乳動物のインスリン様ペプチド 3（INSL3）は性腺由来のホルモンであり、雄では精巣の間質細胞、雌では卵巣の内卵胞膜細胞と黄体細胞でのみ産生されることが知られている。雄動物の末梢血中 INSL3 濃度は、ウシを含む複数の動物種において、精巣機能の変化に応じて変動することが知られているが、雌動物では血中濃度が低いためにその血中動態は不明な点が多い。血中 INSL3 測定には従来、1 種類の抗 INSL3 抗体を用いる競合的免疫測定法が活用されてきたが、2 種類の抗体を用いる高感度なサンドイッチ測定法を雌ウシの血中動態の解析に用いた報告はみあたらない。特に出生子ウシから成牛までの発育過程や卵巣疾患等における雌ウシの血中 INSL3 動態は不明となっている。さらに発情周期中や卵巣疾患のウシにおける卵巣内の INSL3 分泌の変動も解明されていない。

INSL3 は標的細胞の細胞膜表面に存在する INSL3 受容体（リラキシンファミリーペプチド受容体 2 ; RXFP2）を介して作用することが知られている。ウシの卵巣における RXFP2 については、卵胞内の内卵胞膜細胞・卵母細胞や黄体細胞に mRNA の発現が示されているが、卵母細胞の RXFP2 タンパク質の量的解析を行った報告はみあたらない。またウシの精子における RXFP2 タンパク質の発現は凍結精液由来の精子を用いて解析した報告しかみあたらず、新鮮精液の精子を用いて同受容体の発現を調べたものは皆無となっている。

以上のような背景から学位申請者は、第 1 章においては、ウシ INSL3 の高感度サンドイッチ免疫測定法を確立し、それを用いて黒毛和種雌の子ウシから成牛までの年齢群間および種々の繁殖状態における血中 INSL3 動態を解析している。第 2 章では発情周期中と卵胞嚢腫の雌ウシにおける卵巣の主席卵胞および黄体における INSL3 量の変化を調べている。さらに第 3 章では雌ウシの胞状卵胞内卵母細胞および雄ウシの正常および異常精液由来の精子の RXFP2 タンパク質発現を解析している。

第 1 章では申請者は、ウシ INSL3 のサンドイッチ時間分解蛍光免疫測定法を樹立し、従来の競合法に比べて、雌子ウシ等でみられる低濃度の血漿 INSL3 を正確に測定することが可能になったことを示している。申請者はその測定法を用いて雌子ウシから成牛までの血中 INSL3 濃度を測定した。その結果、血中 INSL3 濃度は、子ウシ齢から未経産齢へ、さらに未経産齢から経産齢へと連続的に増加したが、テストステロン濃度は前半の増加はみられたが、後半は変化しなかったことから、産生細胞が同一のテストステロンの分泌パターンとは異なると論じている。一方、発情周期中の卵胞期と黄体期の間および卵胞嚢腫と正常卵胞期の間では INSL3 の濃度差は顕著でなかったことから、雌ウシの末梢血中 INSL3 濃度は発情周期では変動しないことを示唆している。さらに子ウシと経産牛の血中 INSL3 濃度と卵巣の卵胞総容積の間には正の相関がみられるが、テストステロン濃度と卵胞総容積の間には相関はみられないことから、雌ウシの血中 INSL3 濃度は卵胞総容積の指標となる可能性を提唱している。

第 2 章では申請者は、ウシの卵巣内、特に主席卵胞と黄体における INSL3 の局所的な分泌を調べるために、卵胞液と黄体組織の INSL3 量を発情周期の卵胞期と黄体期で比較するとともに、卵胞嚢腫牛の嚢腫卵胞液の INSL3 量を卵胞期のそれと比較した。その結果、卵胞期の主席卵胞 INSL3 濃度と卵巣当りの総量は黄体期に比較して高かったことから、発情時には主席卵胞の INSL3 分泌量は増加すると論述している。また卵胞嚢腫の INSL3 濃度は卵胞期と同程度であるが、テストステロンとエストロジェン濃度は卵胞期よりも低いことから、嚢腫化した卵胞はテストステロン分泌能をすぐに失うが、INSL3 分泌能は維持すると論じている。さらに黄体の INSL3 量は黄体開花期で増加したことから、INSL3 は黄体機能と関連する可能性を述べている。

第 3 章の第 1 節では申請者は、ウシの胞状卵胞の卵母細胞における RXFP2 のタンパク質発現を免疫蛍光組織法により同定した。大型と小型の胞状卵胞間で卵母細胞の同受容体タンパク質量と卵胞液中の INSL3 濃度の比較を行った結果、RXFP2 発現量と INSL3 濃度は大型胞状卵胞よりも小型で高かったことから、INSL3 は小型胞状卵胞の卵母細胞において何らかの役割を果たす可能性があることを論じている。

第 3 章の第 2 節では申請者は、黒毛和種雄ウシから採取した新鮮精液の精子の RXFP2 のタンパク質の発現部位を上述の免疫蛍光組織法で調べて、精子の頭部（先体、赤道節、後先体部）と頚部に発現していることを示した。さらに精子の RXFP2 タンパク質発現量と精漿 INSL3 濃度を、形態が正常な精子の割合が低い（奇形精子が多い）異常精液と正常精子割合の高い（奇形精子が少ない）正常精液の間で比較し、先体、後先体部、異常精液の精子の RXFP2 量は正常精液のそれよりも低く、一方、異常精液の精漿 INSL3 濃度は高いことを見出した。これらの知見から、申請者はウシ精子の受精能獲得において INSL3 は何らかの役割を持つ可能性を論述している。また異常精液の精子では INSL3 の結合部位が減少するため、精漿 INSL3 が余剰となり、INSL3 濃度が高くなる機序を論じている。これらのことから、申請者は精漿 INSL3 濃度が雄ウシの繁殖性を予測するバイオマーカーとなる可能性を提唱している。

以上の研究により、申請者はウシ INSL3 の高感度測定法を樹立して、これまで未解明であった雌ウシの末梢血中 INSL3 動態を明らかにするとともに、その血中濃度は胞状卵胞発育の指標となりうることを示唆した。また性成熟後の雌ウシにおいて INSL3 は卵巣の主席卵胞や黄体内で発情周期に伴って変化することを明らかにした。さらに INSL3 受容体の RXFP2 タンパク質はウシの胞状卵胞の卵母細胞と精子に発現し、その量は変動することから、それら生殖細胞の機能において INSL3 は何らかの役割を演じる可能性を示した。これらの研究成果は、獣医繁殖学領域におけるウシの生殖内分泌学の発展に大きく貢献するものであり、本論文の審査ならびに最終試験の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。