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TITLE:

Demonstrating a Linear Relationship Between Vascular Endothelial Growth Factor and Luteinizing Hormone in Kidney Cortex Extracts

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KEYWORDS:

VEGF, vascular endothelial growth factor, luteinizing hormone, angiogenesis, diabetic nephropathy, bovine kidney

SUMMARY:

Presented here is a protocol for utilizing a cortical kidney extract preparation and total protein normalization to demonstrate the correlation between vascular endothelial growth factor and luteinizing hormone in the mammalian kidney.

ABSTRACT:

Vascular endothelial growth factor (VEGF) helps to control angiogenesis and vascular permeability in the kidney. Renal disorders, such as diabetic nephropathy, are associated with VEGF dysregulation in the kidney. The factors that govern VEGF under physiologic conditions in the kidney are not well-understood. Luteinizing hormone (LH), a pro-angiogenic hormone, helps regulate physiologic VEGF expression in reproductive organs. Given that LH receptors are found in the kidney, it was hypothesized here that LH also helps regulate VEGF expression in the kidney. To provide evidence, we aimed to show that LH levels are able to predict VEGF levels in the mammalian kidney. Most VEGF-related investigations involving the kidney have used lower order mammals as models (i.e., rodents and rabbits). To translate this work to the human body, it was decided to examine the relationship between VEGF and LH in higher order mammals (i.e., bovine and porcine models). This protocol uses the total protein lysate from the kidney cortex. Keys to this method's success include procurement of kidneys from slaughterhouse animals immediately after death as well as normalization of analyte levels (in the kidney extract) by total protein. This study successfully demonstrates a significant linear relationship between LH and VEGF in both bovine and porcine kidneys. The results are reproducible in two different species. The study provides supporting evidence that the use of kidney extracts from cows and pigs are an excellent, economical, and abundant resource for the study of renal physiology, particularly for examining the correlation between VEGF and other analytes.

INTRODUCTION:

Vascular endothelial growth factor A (VEGF-A), helps to regulate angiogenesis and vascular permeability in the kidney and other organs[1,2] (hereafter, VEGF-A will be referred to as VEGF). VEGF levels in the kidney are under tight homeostatic control. When renal VEGF levels are elevated or depressed, the kidney can malfunction. For example, within 3 weeks after birth, mice with podocyte-specific heterozygosity for VEGF develop endotheliosis and bloodless glomeruli (i.e, renal lesions seen in human preeclampsia), and end-stage kidney failure occurs in these heterozygotes by 3 months of age. Podocyte-specific homozygotic knockouts die from hydrops and kidney failure within 1 day of birth[3,4].

On the other hand, overexpression of renal VEGF causes proteinuria and glomerular hypertrophy[3,4]. For example, transgenic rabbits that overexpress VEGF exhibit progressive proteinuria with increased glomerular filtration rates in early stages of nephropathy, followed by decreased glomerular filtration rates in later stages[3]. Diabetic nephropathy, a major cause of end-stage renal disease in diabetic adults, is strongly associated with VEGF dysregulation[2,5]. A great deal of attention has been paid to the role of hypoxia in inducing VEGF expression under pathologic conditions[5]. However, the factors governing VEGF under physiologic conditions (both in the kidney and other organs) are not well-understood[2,6]. Identifying these factors (except for oxygen) that are involved in physiologic and pathologic VEGF regulation is an important undertaking.

Luteinizing hormone (LH), a pro-angiogenic hormone, helps regulate physiologic VEGF expression in reproductive organs such as the ovary and testis[7,8]. Previous studies have provided evidence that LH also helps regulate VEGF in non-reproductive organs, such as the eyes[6,9,10]. LH receptors are found in the medulla and cortex of the kidney[11,12]. Of note, kidney tubular epithelial cells, as well as the LH receptor, express VEGF[11-14]. Taking these two observations together, we hypothesized that LH also helps regulate VEGF expression in the kidney[13,14]. To provide evidence of this LH/VEGF relationship, the presented protocol aims to show that LH levels are able to predict VEGF levels in the kidney. Many previous VEGF-related investigations involving the kidney have used lower order mammal models (i.e., rodents and rabbits)[2]. To translate this work to the human body, the study examines the relationship between VEGF and LH in higher order mammals (here, bovine and porcine models). To carry out this objective, total protein lysate was prepared from the cortex region of bovine and porcine kidneys.

PROTOCOL:

No live or experimental animals were used for this study.

1. Tissue handling

1.1. Procure bovine and porcine whole kidneys immediately after slaughter from an abattoir.

Transport on ice to the laboratory.

1.2. Upon arrival at laboratory, rinse kidneys with 50 mL of ice-cold phosphate buffered saline (PBS). Repeat this step 2x to remove blood completely.

1.3. Keep kidneys on ice (or refrigerated) until further extraction.

2. Dissection of kidneys

2.1. Use sterile scissors, forceps, a knife, and Petri dishes to dissect the kidneys and excise the required tissue portion.

2.2. Prepare RIPA lysis buffer prior to kidney dissection. Dissolve 5 mM NaCl, 0.5 M EDTA, 1 M Tris (pH = 8.0), NP-40 (ID + GEPAL CA-630), 10% sodium deoxycholate, and 10% SDS in double-distilled water, then mix thoroughly. Refrigerate the RIPA lysis buffer when not in use.

2.3. Gently cut the kidney in half (sagittal plane) and cut a piece of tissue (50–70 mm^2) from the cortex region in the center of the kidney (weighing 80–100 mg by wet weight).

2.4. Mince the tissue block into small pieces with a knife to assist the homogenization process.

2.5. After mincing the tissues, transfer them into a microfuge tube with 1 mL of ice-cold 1x RIPA lysis extraction buffer. Place the tubes in ice until further extraction.

3. Tissue homogenization

3.1. Label the microfuge tubes with specific sample details for tissue supernatant collection.

3.2. Using a handheld homogenizer with a sterile probe, then homogenize the tissues for 1–2 min in cold conditions (samples on ice bucket) until no chunks of tissues are visible.

3.3. Subject the tissue extracts immediately for centrifugation in the refrigerated centrifuge at 9,600 *x g* for 5 min at 4 °C.

3.4. Remove the tubes from the centrifuge and place them on the ice bucket.

3.5. Collect the supernatant into a new labeled microfuge tube and store on ice. Discard the pellet.

3.6. Prepare separate aliquots of the supernatants for LH and VEGF-A enzyme-linked immunosorbent assays (ELISA) and total protein analysis, respectively, to avoid freeze-thaw cycles.

4. Bovine and porcine LH ELISA assay

4.1. Store all ELISA assay components included in the commercially available luteinizing hormone (LH) ELISA kit (see **Table of Materials**) at 2–8 °C. This includes the antibody, HRP-conjugate, assay plate (96 well), calibrators, wash buffer (20x concentrate), substrate A, substrate B, and stop solution. Prepare all reagents as recommended by the manufacturer's instructions.

4.2. Before starting the assay, bring all reagents and assay plate to room temperature (RT). Use the required number of wells for the assay, seal, and keep the unused wells at 4 °C until use.

4.3. Dilute the wash buffer (15 mL of 20x concentrate) to 300 mL with double-distilled water

4.4. Set up the blank wells without any solution.

4.5. Add 50 µL of standard or sample to each well (n = 2), then add another 50 µL of horseradish peroxidase (hrp)-conjugate to each well. Immediately add another 50 µL of antibody solution to each well. Seal the plate, mix well, and incubate for 1 h at 37 °C.

4.6. Wash the wells with 1x wash buffer (200 µL/well) and repeat 4x.

4.7. Add 50 µL of substrate A and 50 µL of substrate B to each well, and mix well by tapping the plate on the side gently. Seal the plate and incubate for 15 min at 37 °C in the dark for 15 min.

4.8. Add 50 µL of stop solution to each well, gently tap the plate, and read the plate using the spectrophotometer set to a 450 nm wavelength.

4.9. Normalize bovine and porcine LH levels to total protein (see section 6).

5. Bovine and porcine VEGF-A ELISA assay

5.1. Store all ELISA assay components included in the commercially available Vascular Endothelial Growth Factor-A ELISA kits (see **Table of Materials**) at 2–8 °C. This includes the antibody, HRP-conjugate, assay plate (96 well), calibrators, wash buffer (20x concentrate), substrate A, substrate B, and stop solution. Prepare all the reagents as recommended by the manufacturer's instructions.

5.2. Before starting the assay, bring all reagents and assay plate to RT. Use the required number of wells for the assay, seal, and keep the unused wells at 4 °C until use.

5.3. Dilute the wash buffer (15 mL of 20x concentrate) to 300 mL with double-distilled water

5.4. Add 100 µL of the standard or sample to each well (n = 2). Seal the plate, mix well, and incubate for 2 h at 37 °C.

5.5. Remove the liquid in each well and add 100 µL of detection reagent A to each well, seal the plate, and incubate for 1 h at 37 °C.

5.6. Wash the wells with 1x wash buffer (400 µL/well) and repeat 4x.

5.7. Add 100 µL of detection reagent B to each well and mix well by tapping the plate on the side gently. Seal the plate and incubate the plate for 1 h at 37 °C.

5.8. Wash the wells with 1x wash buffer (400 µL/well) and repeat 4x.

5.9. Add 90 µL of substrate solution to each well, gently tap the plate, and incubate for 1 h at 37 °C.

5.10. Add 50 µL of stop solution to each well, gently tap the plate, and read the plate using the spectrophotometer set to a 450 nm wavelength.

5.11. Normalize bovine and porcine VEGF-A levels to total protein (section 6).

6. Total protein estimation

6.1. Estimate total protein of the bovine and porcine kidney extracts by standard bovine serum albumin (BSA) assay using a commercial kit (see **Table of Materials**) according to the manufacturer's recommendations.

7. Statistical Analysis

7.1. Calculate the mean, median, and standard deviation of each analyte.

7.2. Test the divergence of sample distribution from normal utilizing Kolmogorov-Smirnov Test to decide, upon use, between parametric *vs*. non-parametric statistical tests. If data is normally distributed, then perform statistical testing via parametric tests.

7.3. Under appropriate circumstances (such as normal data distribution), utilize regression models to examine the linear relationship between LH and VEGF-A.

REPRESENTATIVE RESULTS:

The mean and median levels of LH and VEGF by animal type and by sex are shown in **Table 1.** After verifying normality of data by Kolmogorov-Smirnov Testing of normality, linear regression models were utilized to examine the relationship between LH and VEGF. LH was found to be a strong and significant predictor of VEGF in both bovine and porcine kidneys (bovine kidney model: $n = 7$, $R^2 = 0.86$, $p = 0.002$; porcine kidney model: $n = 7$; $R^2 = 0.66$, $p = 0.025$).

The LH/VEGF linear relationship is illustrated in **Figure 1** (bovine regression model) and **Figure 2**

(porcine regression model). The bovine linear equation is as follows: VEGF level = 2.156 x LH level + 68.75. The porcine linear equation is as follows: VEGF level = 196.7 x LH levels + 47.94.

FIGURE AND TABLE LEGENDS:

Table 1: Mean and median LH and VEGF levels by animal type and sex.

Figure 1: LH/VEGF linear relationship in adult bovine kidneys (n = 7).

Figure 2: LH/VEGF linear relationship in adult porcine kidneys (n = 7).

DISCUSSION:

Procuring kidneys from the abattoir immediately after animal death is the key to success in this methodology. This is the main advantage of utilizing organs from cows and pigs instead of human cadavers. There is usually at least a 12–24 h delay from the time of death until human cadaver organs are procured. Because the chemical composition of bodily organs significantly changes within 2 h post-mortem[15,16], VEGF-studies in human cadaver kidneys may not reflect real-life circumstances. Although the protocol greatly emphasizes the importance of immediate procurement and placement of animal organs on ice after extraction, it is not known if other researchers also prioritize this step. For example, the methodology section of a recent study (utilizing bovine and porcine kidneys for the detection of antibiotic residues) did not specify the time delay between animal death and procurement/refrigeration of the organs[17].

This study measures the analytes of interest (VEGF and LH) with commercially available, species-specific ELISAs. ELISAs are highly sensitive, simple to perform assays with, and yield robust results[18]. A critical step in the protocol is the normalization of (ELISA-measured) analyte levels by total protein. The cortical kidney extract is a highly heterogeneous biological substance. In the light of this, a correction factor is essential so that analyte levels can be compared between animals. Thus, normalized by total protein was performed, since we and others have successfully normalized other heterogeneous biological substances (i.e., urine, dried blood spots, and vitreous fluid) in the same manner[9,19,20].

A prior study showed that the correlation between LH and VEGF in vitreous fluid (from bovine and porcine eyes) only manifests after normalization by total protein[6]. Importantly, this normalization step is frequently omitted in published VEGF studies, particularly in those involving ELISA assays. Instead, VEGF levels are often expressed in units such as picogram per milliliter (and not as picogram per milligram of total protein). For example, none of the vitreous VEGF measurements in nine different ELISA studies (that were included in a vitreous VEGF review article) were normalized by any correction method[21,22]. This lack of VEGF normalization in ELISA studies may partially explain why VEGF has not yet been verified as a valid biomarker[21,22].

Despite the limited sample size of the representative data (bovine, n = 7; porcine, n = 7), this

protocol demonstrates a strong and significant linear relationship between LH and VEGF in both bovine and porcine kidneys. That said, there was not a large enough sample size to perform multivariate analyses adjusted for gender. We plan to repeat this study with larger sample sizes so that such analyses can be performed. Nevertheless, the presented results support the potential association between LH and VEGF in the mammalian kidney.

It is expected that this work will help further the understanding of homeostatic regulation of VEGF in the kidney. Both the quality of this methodology and robustness of the findings are illustrated by the reproducibility of the results in two different species. Because animals destined for meat production are healthy, the use of kidney extracts from slaughterhouse animals is primarily for studying physiology; however, their organs are less helpful for studying pathology, which is the main limitation of their use. All in all, the use of renal extracts from cows and pigs are an excellent, economical, and abundant resource for the study of normal adult kidneys. Finally, the protocol demonstrates the effectiveness of utilizing total protein for normalization, particularly when examining correlations between VEGF and other analytes.

ACKNOWLEDGMENTS:
The authors thank Scholl's Slaughterhouse (Blissfield, MI) for providing the bovine and porcine kidneys. No grant funding was utilized for this study.

DISCLOSURES:
Zietchick Research Institute (ZRI) is a private (for-profit) research institute, and Dr. Tammy Movsas (founder and director of ZRI) has a pending patent applications and validated patents for the use of gonadotropin antagonists in the treatment of ocular diseases and diabetes. Other than being an employee (biochemist) at ZRI, Dr. A. Muthusamy has no other financial conflicts to report. A. Arivalagan (summer intern at ZRI, undergraduate student at University of Michigan) has no other financial conflicts to report.

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Figure 1

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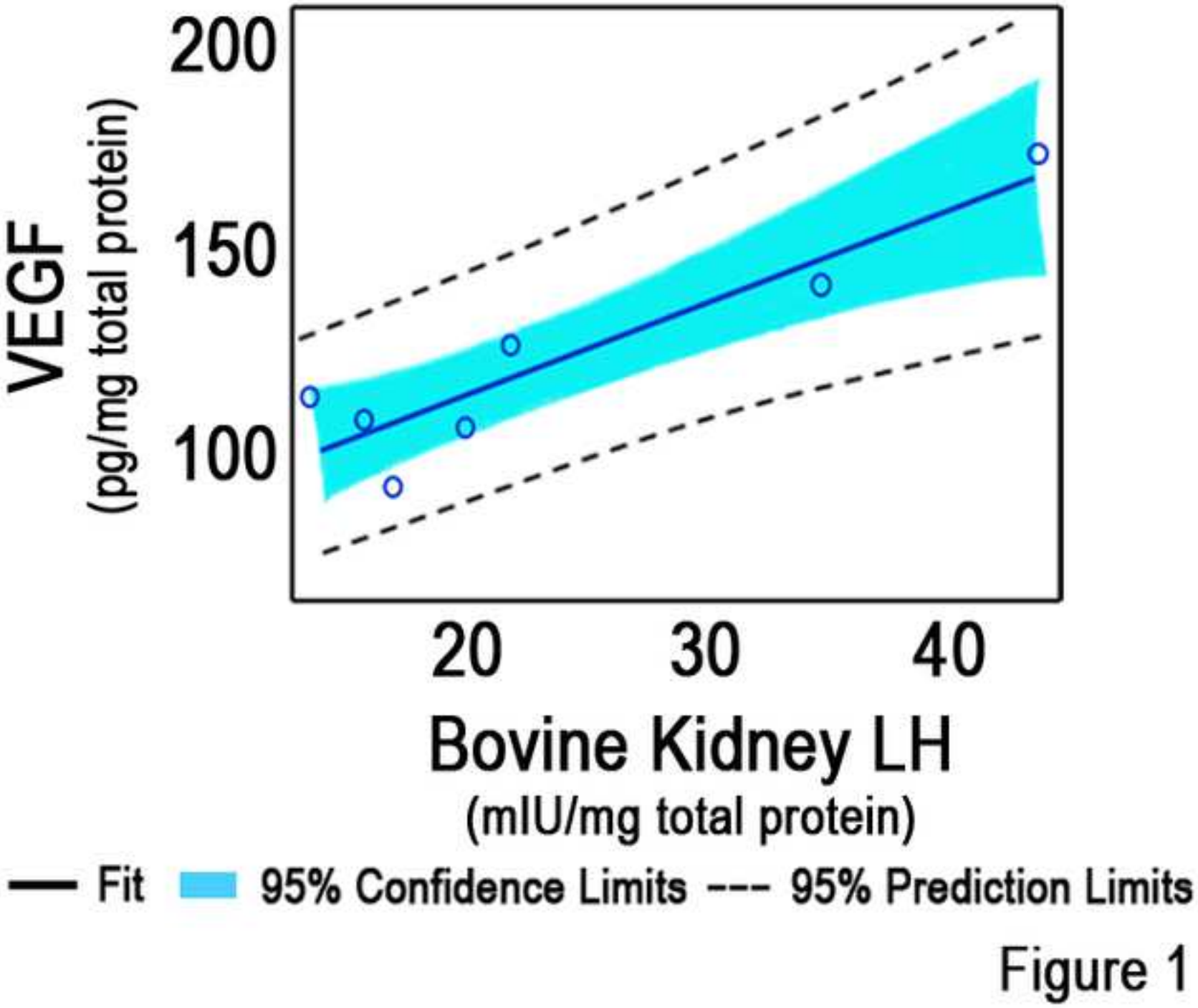

Figure 1

Figure 2

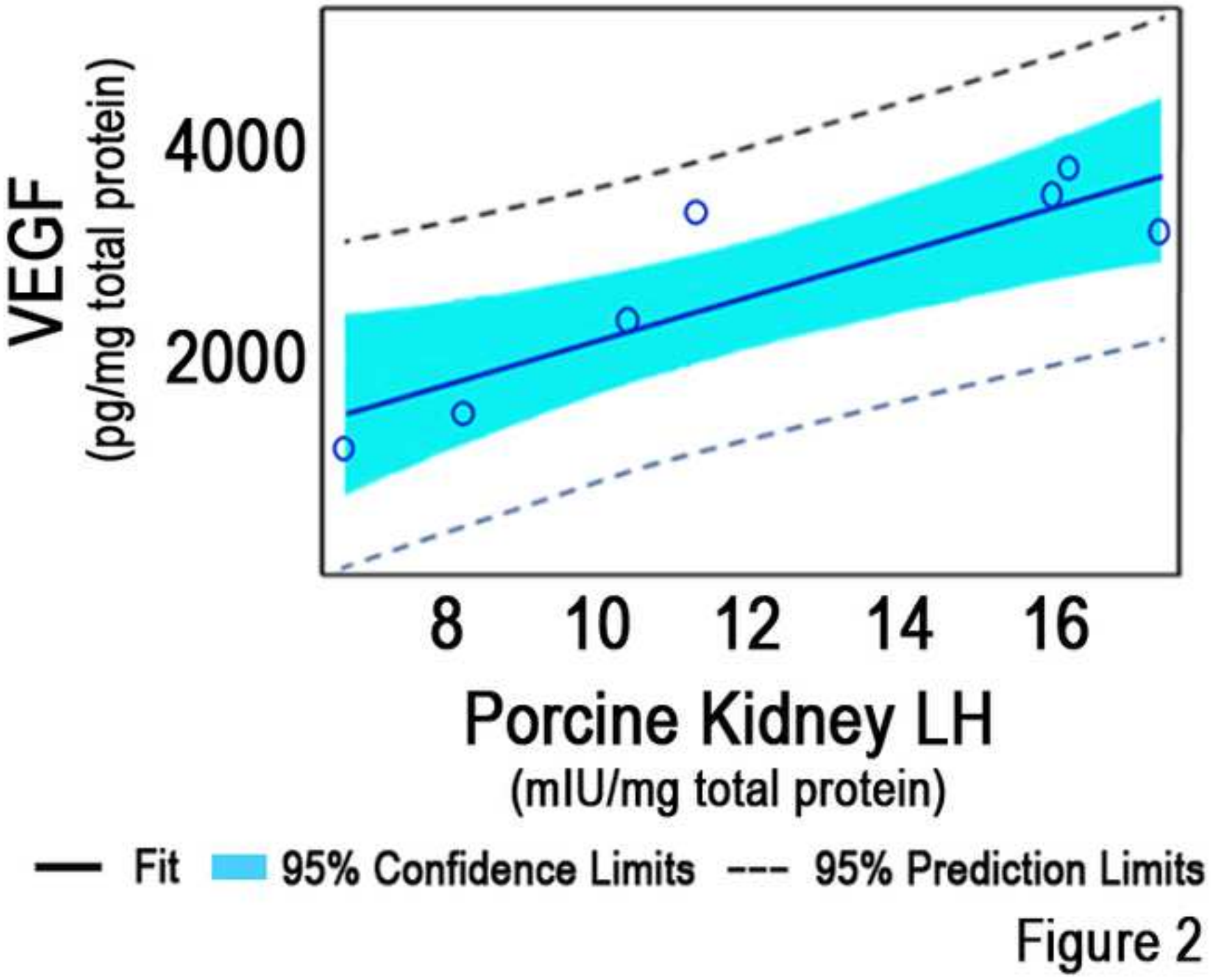

Figure 2

Table 1. Mean and Median LH and VEGF levels by animal type and by sex

Sample Type	Males	Females	All
Bovine Kidneys	N=4	N=3	N=7
LH (mIU/mg total protein)	*Mean:* 27.47 (SD 13.3)	*Mean:* 19.5 (SD 2.1)	*Mean:* 24.06 (SD 10.8)
	Median: 25.7	*Median:* 19.9	*Median:* 19.9
VEGF (pg/mg total protein)	*Mean:* 126.2 (SD 25.8)	*Mean:* 106.0 (SD 14.5)	*Mean:* 120.6 (SD 25.1)
	Median: 131.6	*Median* : 103.5	*Median:* 110.8
Porcine Kidneys	*N=4*	*N=3*	*N=7*
LH (mIU/mg total protein)	*Mean:* 13.2 (SD 3.6)	*Mean:* 12.3 (SD 5.5)	*Mean:* 12.8 (SD 4.5)
	Median: 13.6	*Median:* 10.3	*Median:* 11.2
VEGF (pg/mg total protein)	*Mean:* 2987.2 (SD 772.5)	*Mean:* 2354.1 (SD 932.4)	*Mean:* 2715.9 (SD 901.0)
	Median: 3324.67	*Median* : 2377.3	*Median:* 3226.4

TABLE OF MATERIALS

Name of Material	Company	Catalog Number
Bovine LH ELISA Kit	MyBiosource, San Diego, CA.	MBS700951
Bovine VEGF- A ELISA Kit	MyBiosource, San Diego, CA.	MBS2887434
Micro BCA Protein Assay Kit	ThermoFisher Scientific Inc, Columbus, OH	23235
Porcine LH ELISA Kit	MyBiosource, San Diego, CA.	MBS009739
Porcine VEGF-A ELISA	Ray Biotech, Norcross, GA.	ELP-VEGFA-1
RIPA Lysis and Extraction Buffer	ThermoFisher Scientific Inc, Columbus, OH	89901

We thank the editors for their recommendations. As suggested, we have reworded the passages that too closely resembled the wording in the referenced manuscripts. We have also reworded the title as suggested; the new title is "*Demonstrating a linear relationship between vascular endothelial growth factor and luteinizing hormone in kidney cortex extracts".* We explain below why we do not agree with Reviewer 1 about the need for Western blot data.

Editor's Comment: As asked by reviewer 1, please include a western blot data to show that LH/VEGF and this linear relationship is observed in kidney cortex. This is important as authors emphasize on kidney cortex in the manuscript throughout.

Response: In this study, we utilized commercially-available ELISA assay kits to demonstrate the LH/VEGF relationship in kidney cortical extracts. We did not perform any Western blots (and do not have experience with Western blot methodology). Compared to ELISA, western blots are more time consuming and requires experience to master, especially to achieve unambiguous results. In addition, western blots require optimizing the experimental conditions (i.e. protein isolation, gel concentration, buffers, type of separation). On the other hand, ELISAs are highly sensitive, simple to perform assays with robust results [1]. ELISA is "one of the best validated and most routinely used immunoassay" in protein quantification (such as performed in allergen research).[1] ELISAs have almost completely replaced the former gold standard of protein quantitation-- the radioimmunoassay (RIAs). We certainly do not agree with the reviewer that "serious studies" utilize Western blots (and not ELISAs). Compared to ELISAs, Western blots are often regarded as more of a semi-quantitative test. We do not feel that the lack of Western blot data detracts from our methodology.

We have added the following to our discussion section:

In this study, we measured our analytes of interest (VEGF and LH) with commercially available, species-specific ELISAs. ELISAs are highly sensitive, simple to perform assays with robust results[1]. ELISA is "one of the best validated and most routinely used immunoassay" in protein quantification[1]. A critically important step in our protocol is the normalization of the (ELISA-measured) analyte levels by total protein.

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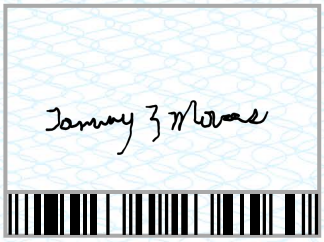

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