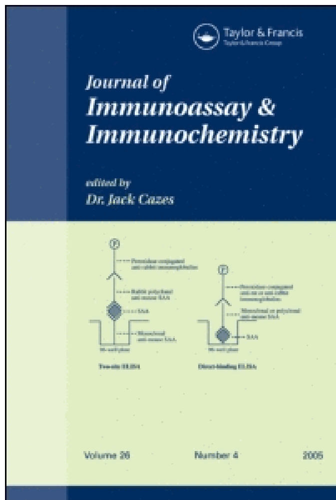
This article was downloaded by:
On: *16 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Generation and Utilization of P450 Cholesterol Side-Chain Cleavage Enzyme and 3β-Hydroxysteroid Dehydrogenase Antibodies for Universal Detection

Chih-Hsien Chiu[a]; Hen-Wei Wei[a]; Leang-Shin Wu[a]
[a] Laboratory of Animal Physiology, Department of Animal Science and Technology, National Taiwan University, Taipei, Taiwan, R.O.C

To cite this Article Chiu, Chih-Hsien , Wei, Hen-Wei and Wu, Leang-Shin(2008) 'Generation and Utilization of P450 Cholesterol Side-Chain Cleavage Enzyme and 3β-Hydroxysteroid Dehydrogenase Antibodies for Universal Detection', Journal of Immunoassay and Immunochemistry, 29: 2, 152 — 160
To link to this Article: DOI: 10.1080/15321810801887839
URL: http://dx.doi.org/10.1080/15321810801887839

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Journal of Immunoassay & Immunochemistry, 29: 152–160, 2008
Copyright © Taylor & Francis Group, LLC
ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810801887839

Taylor & Francis
Taylor & Francis Group

Downloaded At: 10:04 16 January 2011

Generation and Utilization of P450 Cholesterol Side-Chain Cleavage Enzyme and 3β-Hydroxysteroid Dehydrogenase Antibodies for Universal Detection

Chih-Hsien Chiu, Hen-Wei Wei, and Leang-Shin Wu

Laboratory of Animal Physiology, Department of Animal Science and Technology, National Taiwan University, Taipei, Taiwan, R.O.C

Abstract: The biosynthesis of steroids from steroidogenic cells are catalyzed by the two major enzymes, P450 side-chain cleavage enzyme (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD). This article describes the construction of two novel polyclonal antibodies against conserved recombinant protein and the validation of these antibodies on fixed tissue sections of bovine corpus luteum. The polyclonal antibodies were used successfully in Western blots and specifically reacted with P450scc and 3β-HSD protein in bovine luteal cell extracts. Thus, P450scc and 3β-HSD are two specific polyclonal antibodies that are integral products in the investigation of the biological function and regulatory mechanism involved in steroidogenesis.

Keywords: Steroidogenesis, P450scc, 3β-HSD, Polyclonal antibody, Bovine corpus luteum

INTRODUCTION

Steroidogenesis is the process by which specialized tissues synthesize steroid hormones, an important class of terpene-based, small lipid molecules. In the reproductive system, the ovaries and placenta produce progestogens and estrogens which regulate reproductive function and sex characteristics in the female.[1,2] While steroid hormones have diverse functions, they are

Correspondence: Leang-Shin Wu, Laboratory of Animal Physiology, Department of Animal Science and Technology, National Taiwan University, Taipei, Taiwan, R.O.C. E-mail: leshwu@ntu.edu.tw

synthesized via biosynthetic pathways that are initially identical. In all steroidogenic tissues, the initial step in steroidogenesis is the conversion of cholesterol to the first steroid, pregnenolone. This conversion occurs via the action of the cytochrome P450 side-chain cleavage enzyme (P450scc). Pregnenolone then exits the mitochondria and is subsequently converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD).[3,4] Understanding the enzymes involved in steroidogenesis and, more importantly their cellular location, are key factors involved in increasing our knowledge of how the synthesis of steroid hormones is regulated.

Antibodies against P450scc and 3β-HSD will help identify the molecular basis of steroidogenesis in both normal and pathological conditions. Current methods for producing antibodies are not only labor-intensive, but also a rate-limiting step in a myriad of scientific studies. As a result, having widespread availability of antibodies against P450scc and 3β-HSD (among other clinically relevant proteins or enzymes) would be extremely advantageous.[5,6] Therefore, the objective of this study was to develop polyclonal antibodies that could be used to immunologically detect the P450scc and 3β-HSD proteins, regardless of the species of origin.

Downloaded At: 10:04 16 January 2011

EXPERIMENTAL

Production of Recombinant Protein in *E. coli* and its Purification

To create P450scc and 3β-HSD recombinant proteins, the full-length cDNA of P450scc and 3β-HSD (previously verified by recombinant plasmid DNA sequencing) were cloned into the prokaryotic expression vector pET29a. For analysis of the expressed proteins, *E. coli* BL21 (DE3) transformed with the recombinant plasmids, were grown in 2 mL of Luria-Broth (LB) medium containing 50 μg/mL ampicillin (LB/ampicillin) at 37°C for 15 hours. The overnight culture was diluted 1:10 into 5 mL of fresh LB/ampicillin and grown for an additional hour at 37°C. Fusion protein expression was induced by 0.1 mM IPTG, and incubation was continued for 4 hours, The cell culture (1.5 mL) was then immediately pelleted at 12,000 g for 1 minute at 4°C and resuspened in sample buffer for direct SDS-PAGE analysis (according to the method of Laemmli, 1970). Expressed fusion proteins were purified with Ni^{2+}-NTA His-bind resin (Qiagen) and quantified according to the Bradford method (Merck). One aliquot (5 μL) was evaluated for protein purity using a 10% SDS-PAGE.[7]

Production of Antibodies

After purification of the P450scc and 3β-HSD recombinant proteins on Ni^{2+}-NTA His-bind resin, the protein solutions were extensively dialyzed against

phosphate buffer saline (PBS). Four New Zealand white rabbits were immunized (two separate rabbits immunized with each recombinant protein) via subcutaneous injection at multiple sites in the back and flanks, at a dose of 100 μg/animal/injection. For the primary immunization, the protein solutions were mixed with an equal volume of Freunds complete adjuvant. After the primary immunizations, the rabbits were boosted with biweekly injections for two weeks, then two to four times monthly with the P450scc and 3β-HSD recombinant proteins in incomplete Freunds' adjuvant.[8] The final bleeding was performed 10 days following the last injection and the antiserum was stored at −20°C.

Western Blot Analysis

Tissues were collected from the steroidogenic or non-steroidogenic tissues of different species. The protein concentration of the tissue extracts was measured by the Bradford technique and the extracts (20 μg/well) were separated via SDS-PAGE using 12% separating gels under reducing conditions. The proteins were transferred from the separating gels to PVDF membranes (Millipore) using a semi-dry electro-blotting system (Bio-Rad Laboratories, Hercules, CA, USA) for 30 minutes at 16–24 V in a Tris-glycine buffer containing 20% methanol. The membranes were then incubated with diluted polyclonal antiserum (P450scc antiserum: 1:10,000X, 3β-HSD antiserum: 1:40,000X) and developed with a horseradish peroxidase-conjugated secondary antibody directed against rabbit IgG (1:2000X; Santa Cruz Biotech) according to the manufacturer's instructions.

Immunohistochemistry

Samples of bovine corpus luteum tissue were fixed overnight in a 10% formalin-fixed solution, embedded in paraffin, and sectioned at 5 μm. The sections were then mounted on poly-L-lysin coated glass slides. Following deparaffinizing in xylene, tissue sections were rehydrated by passing them through decreasing concentrations of ethanol to Tris-buffer saline. Endogenous peroxidase activity was eliminated by incubating the sections in methanol H_2O_2 for 15 minutes. Nonspecific binding was blocked with normal goat serum, followed by 3 hours incubation with anti-P450scc or anti-3β-HSD antiserum (1:5,000 dilutions). Immunoreactive P450scc or 3β-HSD protein was visualized with an avidin-biotin enhanced horseradish peroxidase method (Vector Laboratories, Burlingame, CA, USA) using diaminobenzidine as the substrate, followed by a light nuclear counterstain with Gill's hematoxylin.

Downloaded At: 10:04 16 January 2011

RESULTS

P450scc and 3β-HSD Recombinant Protein Expression in *E. coli* and its Purification

To express and purify the fusion protein of His-tagged P450scc and 3β-HSD in prokaryocytes, the constructed plasmid was transformed into BL21 (DE3) competent cells, and 0.1 mM IPTG was used to induce the expression of His-tagged P450scc and 3β-HSD protein. The SDS-polyacrylamide gel electrophoresis analysis of crude extracts of recombinant protein demonstrated the presence of the induced expressed P450scc and 3β-HSD proteins (Figures 1A and 1C) with the correct predicted molecular weight. Following purification, the P450scc and 3β-HSD, major bands in the SDS-PAGE could be observed (Figures 1B and 1D). The complete purification procedure resulted in a high protein production level, about 500 μg of His-tagged protein/ 50 mL LB culture.

Downloaded At: 10:04 16 January 2011

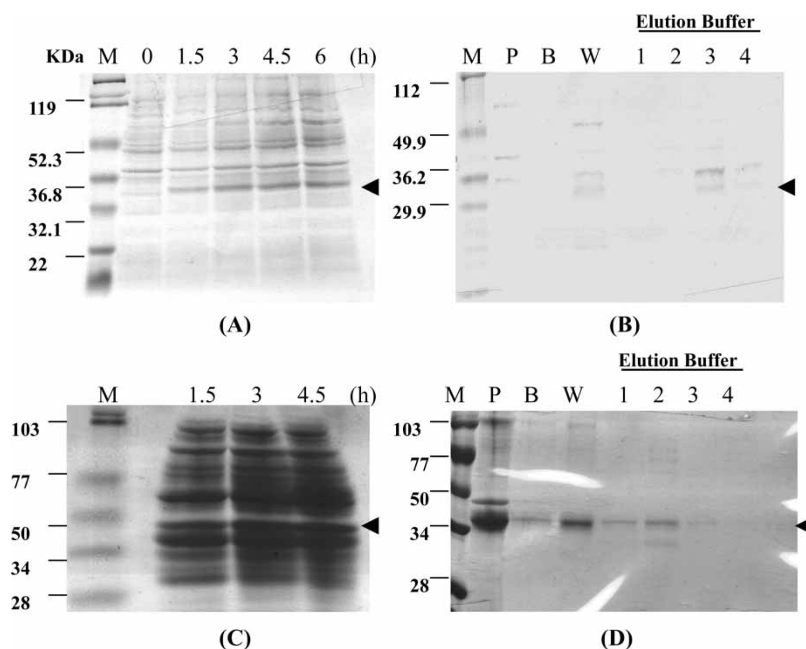


Figure 1. Expression and purification of His-P450scc and His-3β-HSD fusion protein. SDS-PAGE of time-course by IPTG induction in recombinant P450scc (A) or 3β-HSD (C). Purification of recombinant proteins P450scc (B) and 3β-HSD (C). After washing with binding buffer and washing buffer, the purified recombinant protein was eluted with elution buffer, M: marker; P: pass-through; B: binding buffer; W: washing buffer. Each well was loaded with 20 μg total protein of sample.

Antibody Production and Specificity

Initial determinations regarding the specific detection of P450scc or 3β-HSD in animal tissues were achieved using bovine luteal cells incubated with anti-P450scc or anti-3β-HSD antiserum, pre-absorbed and immuno-neutralized with either P450scc or 3β-HSD recombinant protein (1 μg/mL). A single, weak intensity band was detected (Figures 2A and 2B), demonstrating that the antibody was specific and that no additional proteins were detected by the second antibody. Pre-absorption of the antibody resulted in significant reduction or elimination of Western blotting for P450scc or 3β-HSD.

Preliminary findings regarding the titers of the antibodies suggest that the polyclonal antiserum can dilute from 10,000X to 40,000X. Therefore, a high-titer, polyclonal antiserum was produced in the rabbit that recognizes a specific band identified in the luteal cells of bovine (Figures 3A and 3B).

Downloaded At: 10:04 16 January 2011

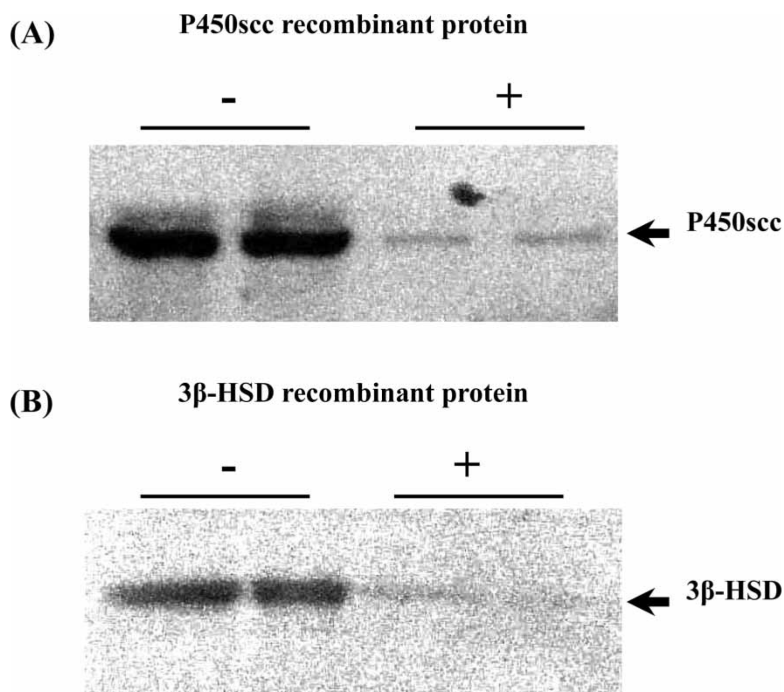


Figure 2. Initial determinations of the specific binding of P450scc (A) or 3β-HSD (B) protein were obtained and verified from bovine luteal cells incubated with anti-P450scc or anti-3β-HSD antiserum pre-absorbed and immuno-neutralized with the immunization recombinant protein (1 μg/mL). Each well was loaded with 20 μg total protein of sample.

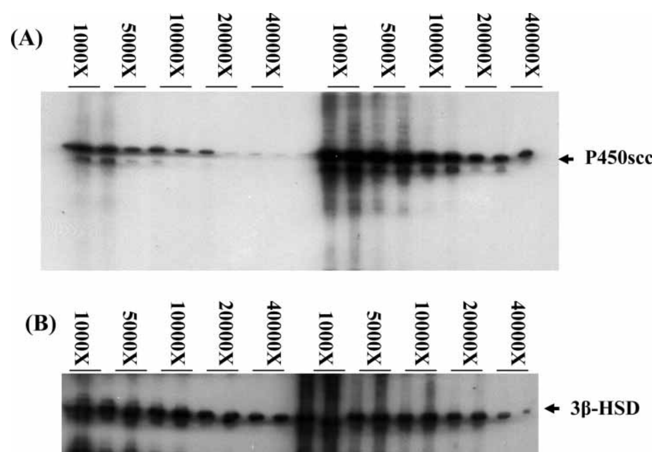


Figure 3. Representative Western blotting for serious dilution (1,000x to 40,000x) of anti-P450scc or anti-3β-HSD polyclone antiserum. Screening lane was loaded with 50 μg total protein of bovine luteal cell lysates.

Downloaded At: 10:04 16 January 2011

Specific Detection of P450scc and 3β-HSD in Steroidogenic Tissue Extracts from Different Species

A single P450scc protein band with an apparent molecular weight of 52 KDa was detected in steroidogenic cell extracts from bovine adrenal (positive control tissue), goat luteal and pig adrenal (Figure 4A). In the anti-3β-HSD Western blot result, a major band with a molecular weight of 43 KDa was detected in steroidogenic cell extracts from a variety of species including the cow, goat, pig, mouse, human, chicken, and duck (Figure 4B).[9,10] The antisera of anti-P450scc and anti-3β-HSD were obtained after immunizing rabbits against the P450scc and 3β-HSD proteins and were tested by Western blot analysis.[11] These results indicated that these two antibodies are highly specific, present high titer values, and have comparable properties.

Immunolocalization of P450scc and 3β-HSD in Bovine Corpus Luteum

Immunoexpression of P450scc and 3β-HSD was detected in the bovine corpus luteum.[12,13] Staining was specific to the cytoplasm of the luteal cells and was not seen in endothelial cells lining the blood vessels (Figures 5A, 5B arrowheads).

The data presented in this report demonstrate that the polyclonal antibodies created against P450scc and 3β-HSD are perfectly suited for

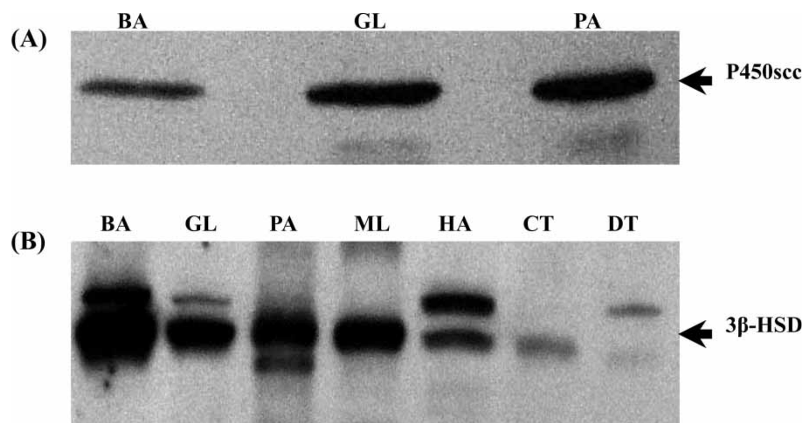


Figure 4. Detection of P450scc (A) or 3β-HSD (B) in the steroidogenic cell extracts from bovine, goat, pig, mouse, human, chicken and duck. On Western blot, a single protein of a molecular weight approximating 52 KDa was recognized by anti-P450scc antiserum (A); A major band approximately 43 KDa was detected by anti 3β-HSD antiserum (B). Extracts were prepared from the following cells: BA, bovine adrenal cells; GL, goat luteal cells; PA, pig adrenal cells; ML, mouse Leydig cells; HA, human adrenal cell line; CT, chicken theca cells; DT, duck theca cells. Each well was loaded with 20 μg total protein of sample.

Downloaded At: 10:04 16 January 2011

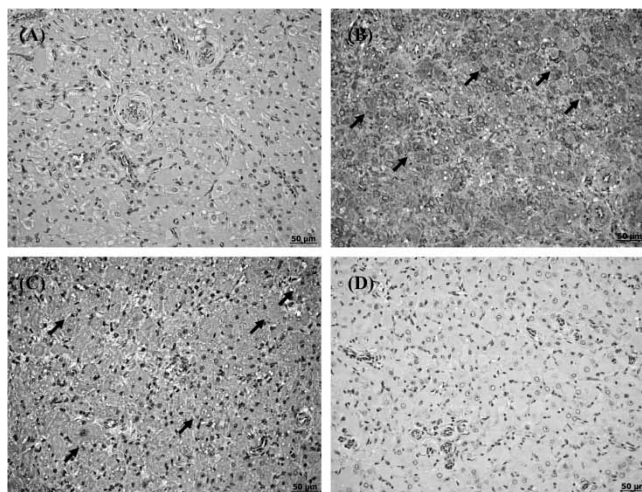


Figure 5. Hematoxylin & Eosin and immunohistochemistry staining of P450scc or 3β-HSD protein in bovine luteum tissue. All slides were photographed at 200X magnification. (A) Hematoxylin and Eosin staining, (B) The P450scc protein was located in luteal cells (arrowheads), (C) The 3β-HSD protein was highly expressed in the luteal cells (arrowheads), (D) Counter staining.

immunohistochemical localization of the P450scc and 3β-HSD in corpus luteum tissue, particularly in endothelial cells.[12,14,15] The results of the Western blots, together with the immunohistochemical assays, indicate that the immunostaining is, indeed, specific to the P450scc and 3β-HSD.

DISCUSSION

In conclusion, we have generated highly specific polyclonal antibodies to the P450scc and 3β-HSD. These two polyclonal antibodies will play an integral role in future elucidation of the cellular and molecular interactions mediating the pleiotypic effects of steroidogenesis in a myriad of species. Indeed, these two antibodies appear to be suitable for both Western blot analysis and immunohistochemistry, allowing for the mapping of these proteins in various steroidogenic tissues and identification of the steroidogenic cells.

Downloaded At: 10:04 16 January 2011

ACKNOWLEDGMENT

The authors gratefully acknowledge the valuable technical advice of Dr. Guo, Department of Veterinary Medicine, National Taiwan University, on the plasmid construction.

REFERENCES

1. Rodway, M.R.; Swan, C.L.; Crellin, N.K.; Gillio-Meina, C.; Chedrese, P.J. Steroid regulation of progesterone synthesis in a stable porcine granulosa cell line: a role for progestins. J. Steroid. Biochem. Mol. Biol. **1999**, *68*, 173–180.
2. Chaffin, C.L.; Dissen, G.A.; Stouffer, R.L. Hormonal regulation of steroidogenic enzyme expression in granulosa cells during the peri-ovulatory interval in monkeys. Mol. Hum. Reprod. **2000**, *6*, 11–18.
3. Bao, B.; Garverick, H.A. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. J. Animal Sci. **1998**, *76*, 1903–1921.
4. Ford, S.L.; Reinhart, A.J.; Lukyanenko, Y.; Hutson, J.C.; Stocco, D.M. Pregnenolone synthesis in immature rat Sertoli cells. Mol. Cell. Endocrinol. **1999**, *157*, 87–94.
5. Sasano, H.; Sasano, N.; Okamoto, M. Immunohistochemical demonstration of cholesterol side-chain cleavage cytochrome P-450 in bovine and human adrenals. Pathol. Res. Pract. **1989**, *184*, 337–342.
6. Lamberski, J.A.; Thompson, N.E.; Burgess, R.R. Expression and purification of a single-chain variable fragment antibody derived from a polyol-responsive monoclonal antibody. Protein Expr. Purif. **2006**, *47*, 82–92.
7. Braunstein, J.D.; Halwer, M. Bradford protein assay and determination of estrogen and progesterone receptors. Clin. Chem. **1986**, *32*, 1588–1589.

8. Verma, N.K.; Sodhi, R.; Rajput, Y.S. Anti-idiotypic antibodies against bovine growth hormone. Asian-Aust. J. Animal Sci. **2003**, *16* (5), 732–737.
9. Kohen, P.; Castro, O.; Palomino, A.; Munoz, A.; Christenson, L.K.; Sierralta, W.; Carvallo, P.; Strauss, J.F., III; Devoto, L. The steroidogenic response and corpus luteum expression of the steroidogenic acute regulatory protein after human chorionic gonadotropin administration at different times in the human luteal phase. J. Clin. Endocrinol. Metab. **2003**, *88*, 3421–3430.
10. Isobe, N.; Nakao, T.; Yoshimura, Y. Distribution of cytochrome P450-side chain cleavage in the theca interna layers of bovine small antral and cystic follicles. Reprod. Domest. Anim. **2003**, *38*, 405–409.
11. Lai, W.W.; Hsiao, P.H.; Guiguen, Y.; Chung, B.C. Cloning of zebrafish cDNA for 3beta-hydroxysteroid dehydrogenase and P450scc. Endocr. Res. **1998**, *24*, 927–931.
12. Conley, A.J.; Kaminski, M.A.; Dubowsky, S.A.; Jablonka-Shariff, A.; Redmer, D.A.; Reynolds, L.P. Immunohistochemical localization of 3 β-hydroxysteroid dehydrogenase and P450 17 α-hydroxylase during follicular and luteal development in pigs, sheep, and cows. Biol. Reprod. **1995**, *52*, 1081–1094.
13. Kobayashi, M.; Nakano, R.; Ooshima, A. Immunohistochemical localization of pituitary gonadotrophins and gonadal steroids confirms the ‘two-cell, two-gonadotrophin’ hypothesis of steroidogenesis in the human ovary. J. Endocrinol. **1990**, *126*, 483–488.
14. Pescador, N.; Soumano, K.; Stocco, D.M.; Price, C.A.; Murphy, B.D. Steroidogenic acute regulatory protein in bovine corpora lutea. Biol. Reprod. **1996**, *55*, 485–491.
15. Fairchild, D.L.; Pate, J.L. Modulation of bovine luteal cell synthetic capacity by interferon-gamma. Biol. Reprod. **1991**, *44*, 357–363.

Received August 5, 2007
Accepted September 18, 2007
Manuscript 3252

Downloaded At: 10:04 16 January 2011