Detection of Retinol-binding Protein in Bovine Yolk Sac, Chorion and Allantois by Immunoperoxidase Method

Kaung Huei Liu*

Department of Veterinary Science. National Chiayi University. Chiayi. Taiwan. ROC

ABSTRACT : Bovine yolk sac at day 24 of pregnancy, and placental membranes (chorion and allantois) from days 70 and 100 of pregnancy were isolated and cultured in a modified minimum essential medium in the presence of [^{35}S]methionine. Proteins synthesized and secreted by isolated bovine yolk sac, chorion and allantois were analyzed by fluorography of two-dimensional polyacrylamide gel electrophoresis. Serum-like proteins,transferrin, α-fetoprotein, α1-antitrypsin and α1-acid glycoprotein ,were the major protein products of yolk sac. A 21 kDa protein produced by yolk sac was identified immunochemically as retinol-binding protein (RBP). Chorion and allantios from days 70 and 100 of pregnancy were active in protein synthesis and secretion. Both chorion and allantois did not secret serum-like proteins but secreted a number of neutral-to-acidic proteins including RBP. Secretory proteins produced by the yolk sac, chorion and allantois may play important roles in the embryonic development and the successful outcome of pregnancy. Antiserum against bovine placental RBP was employed to the immunocytochemistry by immunoperoxidase method. Immunoreactive RBP was localized in epithelial cells and island-like cell clones of yolk sac. Immunostaining for RBP was detected in simple columnar epithelium of chorion and in simple squamous epithelium of allantois. In the present study, proteins synthesized and secreted by yolk sac at day 24 of pregnancy, chorion and allantois from days 70 and 100 of pregnancy were characterized In addition, RBP was localized in yolk sac, chorion and allantois by immunoperoxidase method. The immunoperoxidase method has been proven to be a very effective technique to identify the cellular source of protein synthesis in extraembryonic membranes. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 6 : 783-788)***

Key Words : Bovine, Yolk Sac, Chorion, Allantois, Vitamin A

INTRODUCTION

In bovine, after onset of estrus (day 0), fertilization occurs on day 1 in the oviduct, at the ampullary-isthmic junction (Hunter, 1985). The fertilized egg is surrounded initially by the zone pellucida. By day 4-5, the embryo has developed to the 8-16 cell morula stage and passed from the oviduct into the uterus (Bazer and First, 1983). The compact morula develops into the fluid-filled blastocyst on day 6-8 (Lindner and Wright, 1983). Hatching from the zona pellucida occurs on day 9-10 (Flechon and Renard, 1978). At day 12 of gestation, the hatched blastocyst begins to elongate. Over the next 10 days the blastocyst undergoes a tremendous growth resulting in a filamentous structure.

Endoderm cells spread out from beneath the inner cell mass at about day 8 and completely line trophectodermal cells by day 10 (Massip et al., 1981). Mesodermal cells begin to migrate out from inner cell mass between the trophectoderm and endoderm at days 14-16. The outer layer of mesoderm lines the trophectoderm to form the chorion while the inner layer covers the endoderm to constitute the wall of the yolk sac (Greenstein and Foley, 1958). Allantois is composed of an inner layer of endoderm and an outer layer of vascular mesoderm (Flechon, 1978). By day 22-27, the allantois undergoes a dramatic expansion and fuses with chorion until the chorioallantois is completely formed (Greenstein et al., 1958).

Yolk sac, besides its nutritive function, may play an important role in early embryonic development. Secretory proteins produced by the yolk sac, chorion and allantois may be relevant to the embryonic development and the successful outcome of pregnancy. Proteins produced by bovine conceptuses between days 17-38 of pregnancy (Godkin et al., 1988a), and isolated chorion and allantois between days 29-40 of pregnancy were characterized (Godkin et al., 1988b). Major qualitative alterations in protein synthesis occur between days 24-29, coincident with the development of the allantois and its subsequent fusion with chorion. Days 29-40 chorion and allantois continuously secreted an acidic protein with molecular mass around 21 kDa, previously designated $3B_3$ (Godkin et al., 1988b). The 21 kDa protein has been identified as bovine placental retinol-binding protein (RBP) (Liu et al., 1990). RBP is the specific transport protein for retinol (vitamin A alcohol). Retinol has critical functions in cell differentiation and embryonic development (Gudas et al., 1994). Retinoic acid, a metabolite of retinol, is the only known morphogen in vertebrates (Thaller and Eichele, 1987).

In the present study, proteins synthesized and secreted by isolated bovine yolk sac at day 24 of pregnancy, chorion and allantois from days 70 and 100 of pregnancy were analyzed by fluorography of two-dimensional (2D)

* Address reprint request to Kaung Huei Liu. Tel: +886-5-2717563, Fax: +886-5-2717566, E-mail: arthur@mail.ncyu.edu.tw

Received September 28, 2001; Accepted December 29, 2001

polyacrylamide gel electrophoresis. Patterns of protein electrophoresis of yolk sac, chorion and allantois were compared. The 21 kDa protein produced by yolk sac was identified immunochemically as RBP. In addition, RBP was localized in yolk sac, chorion and allantois by immunoperoxidase method.

MATERIALS AND METHODS

Materials

Eagle's minimal essential medium (MEM) and other supplies for tissue culture were purchased from GIBCO. Supplies for polyacrylamide gel electrophoresis (PAGE) were obtained from Bio-Rad Laboratories. L-[^{35}S]-methionine was obtained from New England Nuclear. Reagents for enzyme immunostaining were obtained from BioGenex. All other chemicals were of reagent grade or better and were products of Sigma Chemical Co.

Animals

Normally cyclic crossbred beef cows were bred by natural service on the day of estrus (day 0). Cattle were slaughtered on day 24 (n=4) following onset of estrus. Uteri with 9.0 cm fetuses (n=4) estimated as day 70 of pregnancy (Bongso and Basrur, 1976) and 20 cm fetuses (n=4) estimated as day 100 of pregnancy (Bongso and Basrur, 1976) were collected from an abattoir. Collected uteri were placed on ice and transported to a laminar flow tissue culture hood.

In vitro culture embryonic tissues

Uteri were opened longitudinally and intact conceptuses were separated from uteri. Yolk sac, chorion and allantois were carefully dissected and cultured with MEM containing [^{35}S]methionine. Incubations were carried out at 37°C in a gaseous atmosphere of 50% N_2, 47.5% O_2, 2.5% CO_2 (by volume) on a rocking platform. After 24 h, incubations were terminated by centrifuging to separate medium from tissue. Conditioned medium samples were dialyzed against 10 mM Tris-HCl buffer, pH 7.6.

Protein electrophoresis

One-dimensional sodium dodecyl sulfate (1D SDS) PAGE was performed according to the method of Laemmli (1970) in 12.5% (w/v) polyacrylamine gels and 5% of stacking gel. The method of Roberts et al. (1984) was used for two-dimensional (2D) SDS-PAGE. Aliquots of dialyzed medium (200,000 cpm) from individual embryonic tissues were lyophilized. Dried samples were dissolved in 75 µl of 5 mM K_2CO_3 containing 9.4 M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol for 2D SDS-PAGE. The proteins were separated by isoelectric focusing in the first dimension and in 12% polyacrylamide gels in the second dimension. Following electrophoresis, Coomassie blue R-250-stained gels were dried after impregnation with 1 M sodium salicylate (Chamberlain, 1979). Fluorographs were prepared and radiolabeled proteins were detected using Kodak film.

Immunoprecipitation

Anti-bovine placental RBP antiserum (50 µl) was added to 1 ml of ^{35}S-label yolk sac tissue culture medium and incubated over night at 4°C. Immune complexes were collected onto protein-A Sepharose CL-4B in 40 mM Tris-HCl (pH 7.5) and solubilized by boiling at 100°C at 5 minutes. Proteins contained in the supernatant were analyzed by 1D SDS-PAGE.

Immunocytochemistry

Yolk sac, chorionic and allantoic tissues were immersed in Bouin's solution for 2 h and washed with water. Tissues were dehydrated in a graded (20%, 40%, 60%, 80% and 100%) alcohol series and embedded in paraffin. Tissue sections (6 µm) were prepared. A modified immunoperoxidase method was used to detect RBP in these issues. Briefly, it consisted of the following steps:

(a) Sections were covered completely with 3% hydrogen peroxide and incubated for 10 minutes at room temperature to block endogenous peroxidase activity.
(b) After washing three times with phosphate buffered saline (PBS), sections were treated with 1% bovine serum albumin for 20 minutes at 37°C. Sections were blotted with paper to remove excess liquid.
(c) After blotting the slides, anti-bovine placental RBP antiserum (Liu et al., 1990) (1:1,000 dilution in PBS), immunoabsorption of the antibody with purified RBP as immune-absorbed serum (negative control) or non-immune serum (negative control) were applied to sections for 30 minutes at 37°C.
(d) After blotting the slides, the link antibody (anti-immunoglobulin serum) was applied to sections for 20 minutes at 37°C.
(e) Sections were washed with PBS and incubated in peroxidase-antiperoxidase solution in PBS with carrier protein (labeling antibody) for 10 minutes at 37°C.
(f) Sections were then washed with PBS and covered with 5% hydrogen peroxide for 10 minutes at room temperature.
(g) Finally, sections were covered with substrate solutions (aminoethylcarbazole or diaminobenzidine) for 10-20 minutes at room temperature.

RESULTS AND DISCUSSION

Day 24 isolated yolk sac connecting with embryo and placental membranes are shown in figure 1. Yolk sac is

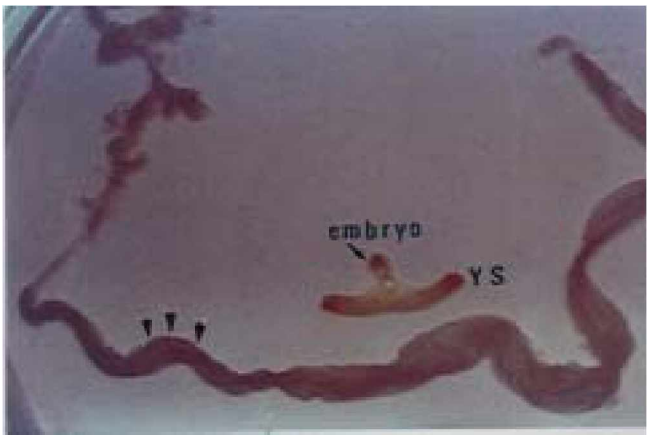

Figure 1. Day 24 isolated yolk sac (YS) connecting with embryo (arrow) and placental membranes consisting with chorion and allantois (arrow heads). ×30.

tubular in shape (2-4 mm×20 mm) with reddish color at both ends. Proteins synthesized and secreted by yolk sac were analyzed by 2D SDS-PAGE (figure 2). Yolk sac specifically produced proteins (label 1-4) which had electrophoretic mobilities and spatial arrangements on 2D SDS-PAGE gels identical to proteins were immunochemically identified in bovine fetal serum as transferrin, α-fetoprotein, α1-antitrypsin and α1-acid glycoprotein respectively (Manabe et al., 1987). This

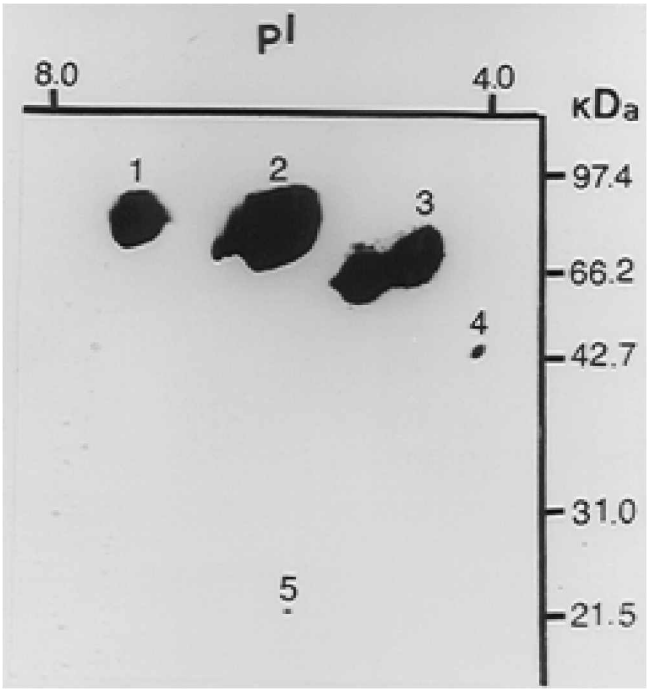

Figure 2. Fluorogram of 2D SDS-PAGE gel for analysis of [^{35}S]methionine-labeled secretory proteins from yolk sac of day 24 of pregnancy. Proteins (200,000 cpm) were loaded onto gel and fluorograph was exposed to dried gel for three weeks.

observation is further supported by the report of Gitlin and Gitlin (1975). They suggested that the yolk sac of human was the source of serum proteins. However immunological identification is necessary before this circumstantial evidence can be ascertained. Transferrin is related with the transport of iron from one part of tissue to another. Iron is a necessary requirement for differentiation of embryonic tissues (Thesleff et al., 1985). Recently, Yun et al. (2001) reported that the expression of insulin-like growth factor-II binding protein-3 in the preimplantation stage is associated with litter size. Thus, iron bound to trasferrin may be considered to be growth factor for rapidly proliferating cells (May and Cuatrecacas, 1985). Iron is also a component of many enzymes, including cytochromes, and flavoproteins (Stryer, 1988) Secretion of transferrin-like protein by yolk sac indicated the need for iron during early embryonic development.

The α-fetoprotein showed a wide pI range suggesting charge heterogeneous of α-fetoprotein molecule. The presence of charge heterogeneity appears to depend on the extent of sialylation of sugar chains (Stryer, 1988). Definitive functions of α-fetoprotein and another α1-acid glycoprotein in embryonic development have not been well understood but some clues concerning the biological roles of carbohydrate units are being uncovered. Carbohydrates conjugated proteins are concerned with molecular targeting and cell-cell recognition. Carbohydrates conjugated proteins have been implicated in cell-cell adhesion during the development of animals, as exemplified by the role of a cell-surface proteoglycan in the aggregation of retinal cells and the adhesion of neurons in the development of the nervous system. Glycoproteins are also components of mucous secretions which act as lubricants in many parts of the body (Stryer, 1988).

The α1-antitrypsin protects tissues from digestion by elastase and trypsin, and blocks the action site of target enzymes (Travis and Salvesen, 1983). Genetic disorders leading to deficient α1-antitrypsin show that this inhibitor is physiologically important. A deficient α1-antitrypsin mutant slowed the secretion of this inhibitor from liver cells. The consequence was that unrestrained excess elastase destroyed alveolar walls in the lungs by digesting elastic fibers and other connective-tissue proteins (Carp et al., 1982). Taken together, these serum-like proteins (transferrin, α-fetoprotein, α1-antitrypsin and α1-acid glycoprotein) are rich in information and are functionally important in early embryonic development .

When the yolk sac has regressed around day 40 of pregnancy, its nutritive function is taken over by chorion and allantois. The electrophoretic patterns of protein synthesis of chorion and allantois at day 70 of pregnancy are shown in figure 3A and 3B, respectively. No changes in patterns of protein synthesis were observed between days

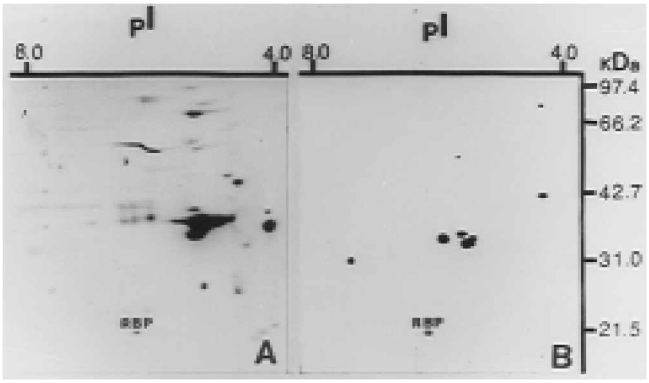

Figure 3. Fluorograms of 2D SDS-PAGE gel for analysis of [^{35}S]methionine-labeled secretory proteins from chorion (A) and allantois (B) of day 70 of pregnancy. Proteins (200,000 cpm) were loaded onto each gel and fluorographs were exposed to dried gels for three weeks.

70 and 100 of pregnancy; therefore, proteins synthesized and released by chorion and allantois from day 70 of pregnancy are respresentive of proteins produced at both days 70 and 100. Neither chorion nor allantois secreted serum-like proteins 1-4 as shown in figure 2. Both chorion and allantois were the source of a number of neutral-to-acidic proteins. Days 40 (Godkin et al., 1988b) through 100 (Fig. 3B) of pregnancy, protein production by allantois remained relatively unchanged. However, several proteins were produced by the chorion at day 40 (Godkin et al., 1988b) that were not observed or barely detectable at day 70 (figure 3A). Although biological functions of most of placental proteins are unclear, proteinaceous products from placental membranes have been reported to have thyrotropic (Avivi et al., 1982), luteotropic (Ailenberg and Shemesh, 1983), antiprostanoid (Knickerbocker et al., 1986), lactogenic (Murthy et al., 1982), and immunosuppressive (Murray et al., 1987) activities. Liu et al. (1990) purified and identified a 21 kDa protein secreted by chorion and allantois as RBP. Additionally, a specific antiserum against purified bovine placental RBP was generated (Liu et al., 1990). The protein 5 presented in figure 2 secreted by yolk sac had electrophoretic mobilities, pI, and spatial arrangement on gel identical to placental RBP secreted by chorion (figure 3A) and allantois (figure 3B) at day 70 of pregnancy. When the bovine placental anti-RBP antiserum was applied to day 24 yolk sac cultures, only single radiolabeled 21 kDa band was immunoprecipitated by 1D SDS-PAGE and fluorography (figure 4, lane 1). The immunoprecipitated 21 kDa protein corresponded to the protein 5 on 2D SDS-PAGE presented in figure 2. Above immunoprecipitation analysis demonstrated that RBP is also a secretory product of bovine yolk sac.

Immunocytochemistry is a useful tool to identify the

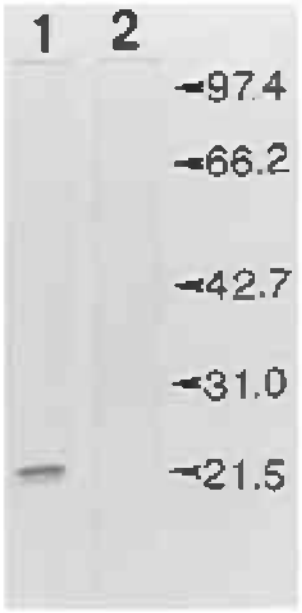

Figure 4. Immunoprecipitation of RBP from yolk sac culture medium followed by 1D SDS-PAGE and fluorography. Exposure time was four weeks.

cellular source of protein synthesis. The gold-labeling methods are used in immunocytochemistry, particularly at the ultrastructural level because the gold particles are easily visible in the electron microscope. If colloidal gold is used as labels at the light microscopical level, the intensity of color may be low under normal illuminating conditions. An epipolarization microscope is necessary to enhance the effect of immunogold (De May, 1983; Goa et al., 1991). Immunoenzyme and immunofluorescence methods are widely used in immunocytochemistry at the light microscopical level. Immunofluorescent preparations must be viewed with a microscope providing light of the correct wavelength to give maximum excitation of the fluorescent label. Immunofluorescent preparations have the disadvantage that they are impermanent and should be photographed within a few days of preparation (Johnson et al., 1982).

In the present study, locations of RBP in yolk sac, chorion and allantois were analyzed by a modified PAP method in the immunocytochemical study. Paraffin-embedded tissue sections of day 24 yolk sac contained epithelial cell sheets and island-like cell clones (arrow) (figure 5). The island-like cell clones are typical for yolk sac. By using placental RBP antiserum and the PAP immunocytochemical system with the substrate chromogen-containing aminoethylcarbazole, the dark stain of sites containing RBP were present in epithelial cell (figure 5A) and island-like cells (figure 5A, arrow). Immunoabsorption of the antibody with purified RBP reduced the staining significantly (figure 5B). The strong

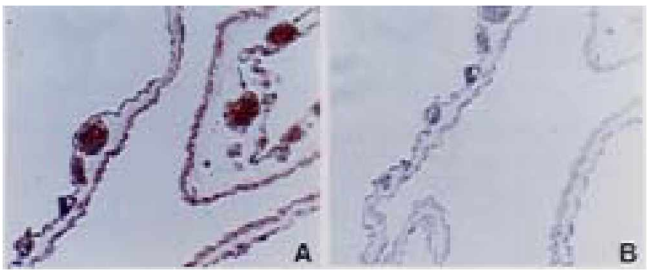

Figure 5. Immunocytochemical localization of RBP in yolk sac of day 24 of pregnancy. Strong immunostaining was in epithelial cells (A) and island-like cell clones (A, arrows) when anti-bovine placental RBP antiserum was applied. Specific immunostaining was significantly diminished or absent in control section (B) when immun-absorbed serum was applied. Panel A, ×60; B, ×60.

immunostaining found in island-like cell clones indicated the major cellular source of RBP in yolk sac. By using placental RBP antiserum and the PAP immunocytochemical system with the substrate chromogen-containing diaminobenzidine, specific brown staining for RBP was detected in simple columnar epithelium of chorion (Fig. 6A) and in simple squamous epithelium of allantois (Fig. 6C). Incubation of tissue sections with non-immune serum did not result in cell staining (figure 6B and 6D). Taken together, the data of fluorograms (figure 2, 3, 4) and immunocytochemistry (figure 5, 6) demonstrated that RBP was synthesized and secreted by bovine yolk sac and the rapidly developing placental membranes (chorion and allantios) through day 100 of pregnancy.

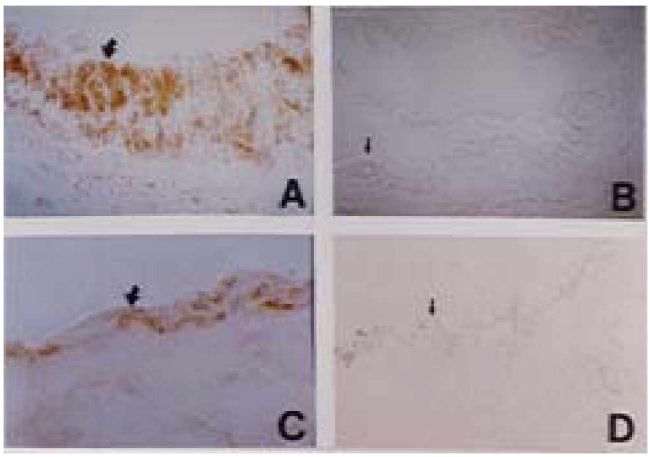

Figure 6. Immunocytochemical localization of RBP in chorion and allantois of day 90 of pregnancy. Strong immunostaining was in epithelial cells of chorion (A, arrow) and allantios (C, arrow) when anti-bovine placental RBP antiserum was applied. Specific immunostaining was absent in respective control sections (B, arrow and D, arrow) when non-immune serum was applied. Panel A, ×180; B, ×60; C, ×180; D, ×60.

The role of RBP of origin of bovine yolk sac, chorion and allantois may involve in the absorption, transport, storage, and metabolism of vitamin A during embryonic development. For example, yolk sac have hematopoietic functions during specific stages of embryonic development for which RBP production may serve to transport the retinol necessary for hematopoietic cell differentiation and proliferation (Amatruda and Koeffler, 1986). Within the placenta, production of RBP by chorion and allantois may provide for the transport of vitamin A from one tissue to another and reducing its rate of metabolism and clearance. In addition, local production of RBP may protect the fetus from free radical damage through the antioxidant properties of retinol (Hiramatsu and Packer, 1990). Given the importance of vitamin A as morphogen, growth factors and signaling molecules (Wolf, 1984), the production of RBP by yolk sac, and chorion and allantois suggested that vitamin A was functionally important in establishment, maintenance, and regulation of pregnancy.

In summary, in the present study, proteins secreted by isolated bovine yolk sac at day 24 of pregnancy as well as chorion and allantois at days 70 and 100 of pregnancy, have been characterized by two-dimensional electrophoresis and fluorography. Yolk sac apparently produced serum-like proteins, transferrin, α-fetoprotein, α1-antitrypsin and α1-acid glycoprotein and RBP. Both chorion and allantios did not secret serum-like proteins but secreted other specific proteins including RBP. The PAP method has been shown to be a very effective technique for identifying the cellular sites of RBP antigens in immunocytochemical study.

ACKNOWLEDGEMENT

The technical assistance of Dr. J. D. Godkin in Department of Animal Science University of Tennessee, Knoxville is gratefully acknowledged.

REFERENCE

Ailenberg, M. and M. Shemesh. 1983. Partial purification of a chorionic gonadotropin-like protein from bovine cotyledons. Biol. Reprod. 28:517-22.

Amatruda, T. T. and H. P. Koeffler. 1986. Retinoids and cells of the hematopoietic system. In: Retinoids and Cell Differentiation (Ed. M. I. Sherman). CRC Press, Boca Raton, FL. pp. 79-103.

Avivi, A., A. B. Schreiber and M. Shemesh. 1982. Isolation and characterization of a 94,000-dalton protein with thyrotropic activity from early bovine placenta. J. Biol. Chem. 257:11384-89.

Bazer, F. W. and N. L. First. 1983. Pregnancy and parturition. J. Anim. Sci. 57:425-46.

Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water soluble fluor sodium salicylate. Anal. Biochem. 98:132-135.

Bongso, T. A. and P. D. Basrur. 1976. foetal fluids in cattle. Can. Vet. J. 17:38-41.

Carp, H., F. Miller, J. R. Hoidal and A. Janoff. 1982. Protential mechanism of emphysema α1-proteinase inhibitor recovered from lungs of cigarette smokers contains oxidized methionine and has decreased elastase inhibitory capacity. Proc. Nat. Acad. Sci. 79:2041-2045.

De Mey, J. 1983. Colloidal gold probes in immunocytochemistry. In: Immunocytochrmistry, Practical Applications in Pathology and Biology (Ed. J. M. Polak and S. V. Noorden). John Wright & Sons, Bristol, pp. 82-112.

Flechon, J. E. 1978. Morphological aspects of embryonic disc at the time of its appearance in the blastocyst of farm mammals. Scanning Electron Microscopy. Vol 2, 54:548.

Flechon, J. E. and J. P. Renard. 1978. A scanning electron microscope study of the hatching of bovine blastocyst in vitro. J. Reprod. Fertil. 53:9-12.

Gao, K., K. H. Liu and J. D. Godkin. 1991. Immunohistochemical localization of bovine placental retinol-binding protein. Int. J. Dev. Biol. 35:485-489.

Gudas, L., M. B. Sporn and A. B. Roberts. 1994. Cellular biology and biochemistry of the retinoids. In: The Retinoids: Biology, Chemistry and Medicine (Ed. M. B. Sporn, A. B. Roberts and D. S. Goodman). New York, Raven Press Ltd. pp. 443-520.

Gitlin, D. and J. D. Gitlin. 1975. Fetal and neonatal development of human plasma proteins. In: The Plasma Proteins: Strucure, Function and Genetic Control (Ed. F. W. Putnum). Academic Press, New York, pp. 977-987.

Godkin, J. D., B. J. Lifsey and G. A. Baumbach. 1988a. Characterization of bovine conceptus protein produced during the peri and postattachment periods of early pregnancy. Biol. Reprod. 38:703-711.

Godkin, J. D., B. J. Lifsey and G. A. Baumbach. 1988b. Characterization of protein production by bovine chorionic and allantonic membranes. Biol. Reprod. 39:195-204.

Greenstein, J. S. and R. C. Foley. 1958. Early embryology of the cow. I. Gastrula and pritive streak stages. J. Dairy Sci. 41:409-421.

Greenstein, J. S., R. W. Murray and R. C. Foley. 1958. Observations on the morphogenesis and histochemistry of the bovine preattachment placenta between 16 and 33 days of gestation. Anat. Rec. 132:321-341.

Hiramatsu, M. and L. Packer. 1990. Antioxidant activity of retinoids. In: Cell Differentiation and Clinical Applications (Ed. L. Packer). Academic Press, Inc. New York. pp. 273-280.

Hunter, R. H. F. 1985. Experimental studies of sperm transport in sheep, cows and pigs. Vet. Rec. 116-188.

Johnson, G. D., R. S. Davidson, K. C. McNamee, G. Russell, D. Goodwin and E. J. Holborow. 1982. Fading of immunofluorescence during microscopy: a stud y of the phenomenon and its remedy. J. Immunol. Methods 55:231-241.

Knickerbocker, J. J., W. W. Thatcher, F. W. Bazer, M. Dorst, D. H. Barron, K. B. Fincher and R. M. Roberts. 1986. Proteins secreted by day 16 to 18 bovine conceptuses extend corpus luteum function in the cow. J. Reprod. Fertil. 77:381-391.

Laemmli, U. K. 1970. Cleavage of structural proeins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lindner, G. M. and R. W. Wright. 1983. Bovine embryo mophology and evaluation. Theriogenology 20:407-416.

Liu, K. H., G. A. Baumbach, P. M. Gillevet and J. D. Godkin. 1990. Purification and characterization of bovine placental retinol-binding protein. Endocrinology 127:2696-2704.

Manabe, T., Y. Takahashi and T. Okuyama. 1987. Identification of bovine fetal and adult serum/plasma proteins by two-dimensional electrophoresis and immunochemical staining. Electrophoresis 8:573-579.

Massip, A., J. Mulnard, R. Huygens, C. Hanzen, P. Van der Zwalmen and F. Ectors. 1981. Ultrastructure of the cow blastocyst. J. Submicrosc. Cytol. 13:31-40.

May, W. S. Jr and P. Cuatrecacas. 1985. Transferrin receptor: its biological significance. J. Membr. Biol. 88:205-215.

Murray, M. .K., E. C. Segerson, P. J. Hansen, F.W. Bazer and R. M. Roberts. 1987. Suppression of lymphocyte activation by a high molecular weight glycoprotein released from preimplantation ovine and porcine conceptuses. Am. J. Reprod. Immunol Microbiol 14:38-44.

Murthy, G. S., C. Schellenberg and H. G. Friesen. 1982. Purifications and characterization of placental lactogen. Endocrinology 111:2117-2228.

Roberts, R. M., G. A. Baumbach, W. C. Buhi, F. B. Denny, L. A. Fitzgerald, S. Babelyn and M. N. Horst. 1984. Analysis of membrane polypeptides by two–dimensional polyacrylamide gel electrophoresis. In: Molecular and Chemical Characterization of Membrane Receptors (Ed. J. C. Ventor and L. C. Harison). Allen R. Liss, Inc., New York. pp. 61-113.

Ordronneau, P., P. B. M. Lindstrom and P. Petrusz. 1981. Four unlabeled anti-body bridge techniques: a comparison. J. Histochem. Cytochem. 29:1397-404.

Sternberger, L. A., P. H. Jr. Hardy, J. J. Cuculis and H. G. Meyer. 1970. The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antiperoxidase) and its use in identification of spirochetes. J. Histochem. Cytochem. 18:315-333.

Stryer, L. 1988. Biochemistry. W. H. Freeman and Company, New York.

Thaller, C. and G. Eichele. 1987. Identification and spatial distribution of retinoids in the developing chick limb bud. Nature 327:625-628.

Thesleff, I., A. M. Partanen, W. Lanshulz , I. S. Trowbridges and P. Ekblom. 1985. The role of transferring receptors and iron delivery in mouse embryonic morphogenesis. Differentiation 30:152-158.

Travis, J. and G. S. Salvesen. 1983. Human plasma proteinase inhibitors. Ann. Rev. Biochem. 52:655-709.

Yun, J. S., W. J. Kang, D. S. Seo, S. S. Park, K. C. Hong, C. Y. Lee and Y. Ko. 2001. Association of Endocrine factors (insulin-like growth factor-II and binding protein-3) with litter size in pigs. Asian-Aust. J. Anim. Sci. 14:307-315.

Wolf, G. 1984. Multiple functions of vitamin A. Physiol. Rev. 64:873-937.