ACTA VET. BRNO, 56, 1987: 487-493

A TECHNIQUE FOR THE DEMONSTRATION OF IMMUNOGLOBULINS IN THE CUMULUS OOPHORUS-OOCYTE COMPLEX OF THE PIG

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Received March 10, 1987

Abstract

Trávník P., D. Horký: A Technique for the Demonstration of Immunoglobulins in the Cumulus oophorus-Oocyte Complex of the Pig. Acta vet. Brno, 56, 1987: 487-493.

An optimized procedure for the demonstration of immunoglobulins in the pig cumulus oophorus-oocyte complex in semi-thin sections is described. Non-specific porcine immunoglobulins of class G were demonstrated by means of peroxidase-labelled goat antiserum, and specific immunoglobulins of class G against Aujeszky's disease virus were demonstrated using the corresponding antigen and peroxidase-labelled porcine antibodies against this antigen.

A most convenient mode of removing the embedding medium was developed, the composition of the incubation solution for the demonstration of peroxidase activity was improved and a number of checks on the specificity of the reaction were tested. The procedure proved most useful for the demonstration of specific and non-specific immunoglobulins both in oocytes and in the cumulus oophorus.

Oocyte, cumulus oophorus, immunoglobulins, immunocytochemistry, semi-thin sections.

Light microscopic examination of semi-thin sections provides sufficient information on the location of a substance as to whether it is present in the cumulus oophorus cells, intercellular spaces of the cumulus, in the zona pellucida, perivitelline space, cytoplasm or in the nucleus of the oocyte. Moreover, semi-thin sections of the cumulus-oocyte complex are easier to prepare than traditional paraffin-embedded sections. In the light of these considerations we chose the immunocytochemical demonstration of specific and non-specific immunoglobulins in semi-thin sections of the pig cumulus-oocyte complex as the basic method of their

localization. In developing the procedure of immunocytochemical demonstration of antigens in the mammalian cumulus-oocyte complex our main objectives were: optimization of the procedure for removal of the embedding medium, ensurance of the specificity of the immunocytochemical reaction and optimization of the composition of the incubation solution for the demonstration of peroxidase activity.

Materials and Methods

Freshly collected oocytes were fixed with 300 mmol/l formaldehyde (prepared from paraformaldehyde) at room temperature for 1 to 2 h and then washed in cacodylate buffer (pH 7.3, 130 mmol/l) with a saccharose supplement at 0 to 4°C till the following day. They were either postfixed for 0.5 h with osmium tetraoxide (40 mmol/l) in cacodylate buffer (pH 7.3, 130 mmol/l) with subsequent washing in cacodylate buffer (pH 7.3, 130 mmol/l) or left without postfixation.

The oocytes were then dehydrated in a series of ethanol of ascending concentration, saturated with Durcupan ACM and embedded in the same medium. Sections, 500 nm thick, were prepared from blocks with embedded oocytes and heat-fixed onto slides.

The embedding medium was removed from the sections with a solution of sodium methanolate (500 mmol/l) for 5 minutes and the sections were then washed with absolute alcohol and transferred to bidistilled water using a series of ethanol of descending concentration. Afterwards the sections were treated with hydrogen peroxide (300 mmol/l) to remove osmium compounds from postfixed preparations and to inhibit endogenous peroxidase activity and subsequently rinsed in bidistilled water and buffered saline containing human serum albumin (10 g/l).

Further treatment of the sections proceeded along three different lines with respect to the following objectives:

a) For demonstration of the presence of immunoglobulins in oocytes, peroxidase-conjugated goat immunoglobulin against pig immunoglobulin of class G was used. It was applied for 1 h in a 1:10 or 1:20 dilution with phosphate-buffered saline or phosphate-buffered saline supplemented with 500 mmol/l sodium chloride. The controls were sections preincubated with unlabelled goat immunoglobulin against pig immunoglobulin of class G in the dilution of 1:10.

b) For demonstration of the presence of immunoglobulins against Aujeszky's disease (AD) virus, the sections were first incubated for 1 h with a suspension of viral antigen diluted 1:10. Afterwards peroxidase-conjugated pig antiviral immunoglobulin was applied for 1 h in a 1:10 or 1:20 dilution of buffered saline or buffered saline supplemented with 500 mmol/l sodium chloride. The controls were sections of oocytes from non-immunized animals and sections treated with pig immunoglobulin against AD virus in the 1:10 dilution with buffered saline without being previously treated with the virus.

c) Sections to be used as checks on the specificity of peroxidase demonstration were incubated only with non-conjugated goat immunoglobulin against pig immunoglobulin of class G in a 1:10 dilution with buffered saline.

In all three cases the sections were washed with buffered saline containing human albumin (10 g/l) between the individual antibody applications and after the incubation was completed. Afterwards the sections were washed with bidistilled water and incubated for the demonstration of peroxidase. This incubation was carried out in a solution containing 1.4 mmol/l 3.3´-diaminobenzidine (tetrahydrochloride), 6 mmol/l trishydroxymethylaminomethane and 3 mmol/l hydrogen peroxide prepared from a semi-product according to an innovation scheme (ZN 41/86 J. E. Purkyně University, Trávník and Horký). After incubation at room temperature for 15 minutes the sections were washed with bidistilled water and part of them were treated with osmium tetroxide (40 mmol/l) to make the reaction product more pronounced. After being washed again, the sections were mounted in glycerol or Entellan, examined and photographed.

Results and Discussion

A prerequisite for a successful performance of immunocytochemical reaction in semi-thin sections is the fixation that preserves the structure of the cells and exerts a minimum effect on antigenic determinants. In our study we chose two types of fixation: single fixation with formaldehyde and double fixation with formaldehyde and osmium tetroxide. In both cases the structure of the cells at the light microscopy level was very good (Fig. 1 and 2). Sternberger (1979) recommended formaldehyde as a fixative causing relatively little distortion of antigenic structure. Litwin et al. (1984) reported good experience with formaldehyde in the immunocytochemical demonstration of antigens in semi-thin sections. On the other hand, reservations exist as to the use of glutaraldehyde which inhibits the immunocytochemical reactivity particularly of immunoglobulins (Rodning et al. 1980; Takamiya et al. 1980) and pituitary hormones (Batten and Hopkins 1978; Tougard et al. 1980). Our results confirmed the usefulness of formaldehyde also with regard to the intensity of immunocytochemical reaction. Postfixation with osmium tetroxide in part of the sections in our study was adopted because cumulus-oocyte complexes are so small objects that after embedding it is difficult to look them up in the block if post-fixation has not been applied. From the results reported by Litwin et al. (1984) it appears that immunocytochemical reaction can be carried out successfully in semi-thin sections after postfixation with osmium tetroxide provided that antigenic determinants have been released by oxidation with hydrogen peroxide (Nakane 1971) or possibly with other intense oxidizing agents. In our study the postfixation with osmium tetroxide proved satisfactory: the intensity of the reaction was good. Increased staining of the background as described after fixation with osmium tetroxide by Litwin et al. (1984) was not observed. This may be associated with the fact that the aforementioned investigators used 90-minute fixation in a 2% solution of osmium tetroxide, whereas in our study the fixation was carried out with a 1% solution of osmium tetroxide and only for 30 minutes.

Preparations of oocytes non-postfixed with osmium tetroxide showed a markedly higher intensity of reaction in the demonstrat-

ion of both specific and non-specific immunoglobulins. The amount of the reaction product in follicular cells was lower than in oocytes (Fig. 1). This result is in keeping with findings of reduced binding of antibodies to sections postfixed with osmium tetroxide as compared with single aldehyde fixation (Litwin et al. 1984).

An important factor affecting the quality of immunocytochemical reaction is the mode in which the embedding medium is removed. In the literature there are reports on the use of sodium ethanolate (Lackie et al. 1985; a.o.) which, however has the drawback of producing rapid damage to the structure of the cells. Another approach is based on the use of a solution of sodium hydroxide in ethanol (Litwin et al. 1984; a.o.) which, however, must be allowed to act for at least 45 to 60 minutes whereby the possibility of damage to antigenic determinants is increased. In our study it was a solution of sodium methanolate (Mayor et al. 1961) that proved most useful: it acts very quickly (5 minutes) and perfectly removes the embedding medium without damaging the structure of the cells as was also shown in a scanning electron microscope study (Trávník et al. 1982).

To enhance the binding of antibodies to antigenic determinants in sections, a number of investigators used mild digestion with proteolytic enzymes as suggested by Hautzer et al. (1980). However, Dell´Orto et al. (1982) found that protease digestion exerted a marked effect on the immunocytochemical reactivity of tissues fixed with glutaraldehyde but had little, if any, effect in samples fixed with formaldehyde or other fixatives. Therefore treatment of the sections with trypsin or other proteolytic enzymes was not tested in our study.

Considering that endogenous peroxidase or pseudoperoxidase activity may be an obstacle to the use of immunoperoxidase techniques it appears desirable that peroxidase activity in the sections should be blocked before further treatment. This question was discussed in detail by Matsumoto (1985). In our study the sections were exposed to the action of methanol and hydrogen peroxide, a procedure ensuring sufficient inhibition of the endogenous activity (Streeferk 1972). Control sections showed no endogenous peroxidase activity.

For immunocytochemical demonstration of specific and non-specific immunoglobulins, antigens and antisera were prepared with standardized techniques (Rodák 1984; Rodák et al. 1985). They were used in relatively high titres of 1:10 and 1:20 which, in terms of the order, are in keeping with those generally used for immunocytochemical demonstration of antigens in semi-thin sections (Litwin et al. 1984). No difference was found in the intensity of reaction between the two titres.

Sufficient evidence of the specificity of the binding of immunoglobulins upon visualization in semi-thin sections is generally seen in the control reaction with a non-specific immunoglobulin (Tuczek et al. 1985). In our study these checks were extended to include preincubation of the sections with the corresponding non-labelled antibodies before incubation was carried out in antiserum of increased sodium chloride concentration. According to Grube and Weber (1980) the use of antiserum containing 0.5 mol/l sodium chloride does not exert a substantial effect on the specific binding of immunoglobulins to antigen but inhibits

non-specific interactions. All the checks showed that non-specific binding of immunoglobulins to the structures of the cumulus-oocyte complex did not occur.

For the demonstration of bound peroxidase-labelled immunoglobulins we used a modification of the method described by Graham and Karnovsky (1966). In doing so we were guided by the observations of Vacca et al. (1978) that indicate the suitability of a lower pH in the demonstration of peroxidase. Our modification of the medium based on lowering the pH to 5.7 and on a minimum content of buffer (trishydroxymethylaminomethane) proved quite satisfactory with respect to both visualization of the enzyme reaction and stability of the incubation solution. To save time and effort in preparing the embedding medium and reduce hazards involved in handling carcinogenic diaminobenzidine, we used dry half-finished medium kept under conditions of long-term storage in the dark at reduced temperature. This approach seems to us more convenient than the use of a frozen concentrate (Mauro et al. 1985).

To make the reaction product more pronounced, we used additional binding of osmium tetroxide in part of the samples. This treatment distinctly increased the contrast of the reaction product, with no staining of the background being observed. Stained were lipid droplets which constitute constant part of the cytoplasm of pig oocytes (Fig. 2). However, lipid droplets are easy to differentiate from other cell structures and their staining facilitated the detection of oocytes in control sections where otherwise oocytes were not well perceptible under the microscope because of the absence of the reaction product.

It can be concluded that the technique of immunocytochemical demonstration of antigens in semi-thin sections of the cumulus-oocyte complexes described in the present study yielded standard results. It may prove useful in the demonstration not only of immunoglobulins but also of other components of oocytes, follicular cells and other structures contained in the cumulus-oocyte complex.

Metoda průkazu imunoglobulinů v komplexu kumulus-oocyt prasete

V práci je popsán optimalizovaný postup průkazu imunoglobulinů v komplexu kumulus-oocyt prasete v polosilných řezech. Byly prokazovány nespecifické prasečí imunoglobuliny třídy G pomocí kozího antiséra značeného peroxidázou a specifické imunoglobuliny třídy G proti viru Aujeszkyho choroby (ACH) pomocí příslušného antigenu a prasečích protilátek proti tomuto antigenu značených peroxidázou.

Byl vypracován nejvhodnější způsob odstraňování zalévacího media, zdokonaleno složení inkubačního roztoku pro průkaz peroxidázové aktivity a ověřena řada kontrolních přístupů pro zjištění specifity reakce. Popsaný postup se ukázal jako plně vhodný pro průkaz specifických i nespecifických imunoglobulinů v oocytech i v cumulus oophorus.

Метод определения иммуноглобулинов в комплексе холмик-ооцит свиней

В работе дается описание оптимизованного метода определения иммуноглобулинов в комплексе холмик-ооцит свиньи в полусильных цепях. Были установлены неспецифические свиные иммуноглобулины класса G с помощю козьей антисыворотки, меченой пероксидазой, и специфические иммуноглобулины класса G против вируса Ауески (БА) с помощю соответствующего антигена и свиных антител против данного антигена, меченых пероксидазой.

Был разработан самый приемлемый метод удаления вливаемого вещества, совершенствован состав инкубационного раствора для определения активности пероксидазы и проверяли ряд контрольных методов для определения специфичности реакции. Приведенный метод оказался весьма пригодным для определения специфических иммуноглобулинов в ооцитах и в холмике - яйценосном холмике.

References

BATTEN, T. C. - HOPKINS, C. R.: Discrimination of LH, FSH, TSH and ACTH in dissociated porcine anterior pituitary cells by light and electron microscope immunocytochemistry. Cell Tissue Res., 192, 1978: 107-120.

DELL´ORTO, P. - VIALE, G. - COLOMBI, R. - BRAIDOTTI, P. - COGGI, G.: Immunohistochemical localization of human immunoglobulins and lysozyme in epoxyembedded lymph nodes. Effect of different fixatives and of proteolytic digestion. J. Histochem. Cytochem, 30, 1982: 630-636.

GRAHAM, R. C. - KARNOVSKY, M. J.: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem., 14, 1966: 291-302.

GRUBE, D. - WEBER, E.: Immunoreactivities of gastrin (G)- cells II. Nonspecific binding of immunoglobulins to G-cells by ionic interactions. Histochemistry, 66, 1980: 149-167.

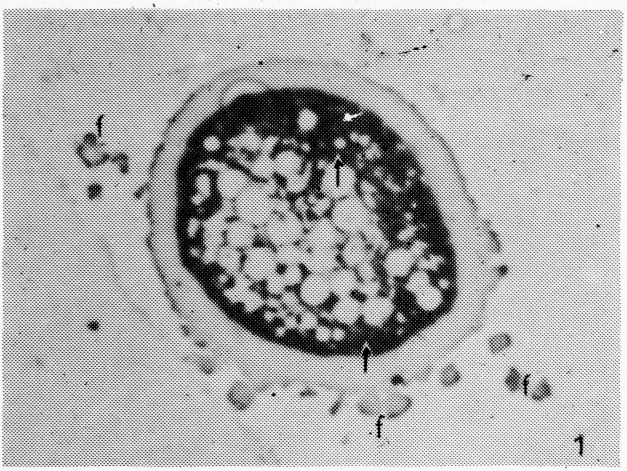
HAUTZER, N. W. - WITTKUHN, J. F. - McCAUGHEY, W. T. E.: Trypsin digestion in immunoperoxidase staining. J. Histochem. Cytochem., 28, 1980: 52-53.

LACKIE, P. M. - HENNESSY, R. J. - HACKER, G. W. - POLAK, J. M.: Investigation of immunogold-silver staining by electron microscopy. Histochemistry, 83, 1985: 545-550.

LITWIN, J. A. - YOKOTA, S. - FAHIMI, H. D.: Light microscopic immunocytochemical demonstration of peroxisomal enzymes in Epon sections. Histochemistry, 81, 1984: 15-22.

MATSUMOTO, Y.: Simultaneous inhibition of endogenous avidin-binding activity and peroxidase applicable for the avidin-biotin system using monoclonal antibodies. Histochemistry, 83, 1985: 325-330.

MAURO, A. - GERMANO, I. - GIACCONE, G. - GIORDANA, M. T. - SCHIFFER, D.: 1-Naphthol basic dye (1-NBD). An alternative to diaminobenzidine (DA B) in immunoperoxidase techniques. Histochemistry, 83, 1985: 97-102.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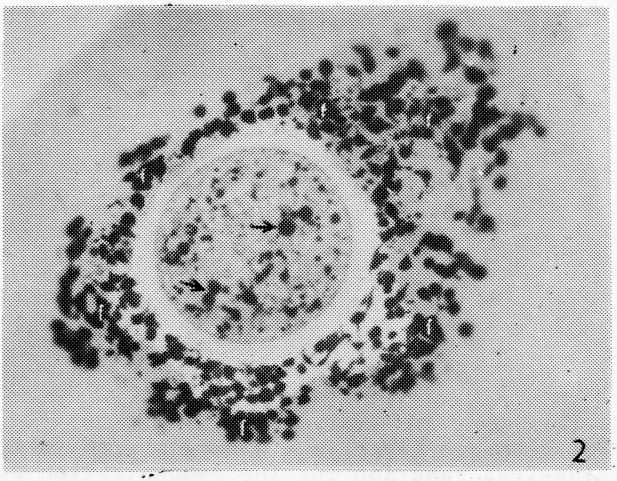
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Fig. 1. A semi-thin section of a pig oocyte with follicular cells. Immunocytochemical reaction for the demonstration of immunoglobulins; the preparation was not postfixed with osmium tetraoxide. Intense positive reaction in the cytoplasm of the oocyte / ——— / and on the surface of the zona pellucida / ——— /. A smaller quantity of the reaction product in follicular cells /f/. Magnification: 450x.

Fig. 2. A semi-thin section of a pig cumulus-oocyte complex. A large quantity of the reaction product can be seen in follicular cells /f/. Positive reaction is shown by the cytoplasm of the oocyte; no reaction product in the zona pellucida. Non-specific staining of lipid droplets in the cytoplasm of the oocyte / ——— / is due to the binding of osmium tetraoxide. Magnification: 250x.

MAYOR, H. D. - HAMPTON, J. C. - ROSARIO, B.: A simple method for removing the resin from epoxy embedded tissue. J. Cell Biol., 9, 1961: 909-910.
NAKANE, P. K.: Application of peroxidase-labelled antibodies to the intracellular localization of hormones. Acta Endocrinol. (Copenh.) (Suppl), 153, 1971: 190-204.
RODÁK, L.: Application of radioimmunological methods for checking the quality of class-specific antibodies against bovine and porcine immunoglobulins. Veterinary Immunology and Immunopathology, 5, 1983/1984: 377-387.
RODÁK, L. - ŠMÍD, B. - VALÍČEK, L. - JURÁK, E.: Collection of microvolume blood samples into glass capillaries for the detection of antibody against Aujeszkys disease virus in pigs by enzyme-linked immunosorbent assay (ELISA) and solid-phase radioimmunoassay (RIA). Acta vet. Brno, 54, 1985: 207-216.
RODNING, C. B. - ERLANDSEN, S. L. - COULTER, H. D. - WILSON, I. D.: Immunohistochemical localization of IgA antigens in sections embedded in epoxy resin. J. Histochem. Cytochem., 28, 1980: 199-205.
STERNBERGER, L. A.: Immunocytochemistry, 2nd edn. John Wiley and Sons, New York, 1979.
STREEFKERK, J. G.: Inhibition of erythrocyte pseudoperoxidase activity by treatment with hydrogen peroxide following methanol. J. Histochem. Cytochem., 20, 1972: 829-831.
TAKAMIYA, H. - BATSFORD, S. - VOGT, A.: An approche to postembedding staining of protein (immunoglobulin) antigen embedded in plastic: prereguisites and limitations. J. Histochem. Cytochem., 28, 1980: 1041-1049.
TOUGARD, C. - PICART, R. - TIXIER-VIDAL, A.: Immunocytochemical localization of glycoprotein hormones in the rat anterior pituitary. A light and electron microscope study using antisera against rat - subunits: a comparison between pre-embedding and post-embedding methods. J. Histochem. Cytochem., 28, 1980: 101-114.
TRÁVNÍK, P. - OMELKOVÁ, A. - PROCHÁZKA, V.: Use of Tesla BS 300 scanning electron microscope for examination of semi-thick section of mammalian embryos. Kovoexport, 4, 1982: 10-12.
TUCZEK, H. V. - FRITZ, P. - GRAU, A. - MISCHLINSKY, A. - WAGNER, T. - WEGNER, G.: Distribution of albumin in normal and regenerating livers of mice. A light microscopic immunohistochemical and autoradiographic study. Histochemistry, 83, 1985: 165-169.
VACCA, L. L. - HEWETT, D. - WOODSON, G.: A comparison of methods using diaminobenzidine (DAB) to localize peroxidases erythrocytes, neutrophils, and peroxidase. Stain Technol., 53, 1978: 331-336.