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REGULAR ARTICLE

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# Immunohistochemical localization of zona pellucida proteins ZPA, ZPB and ZPC in human, cynomolgus monkey and mouse ovaries

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**Abstract** The zona pellucida of mammalian oocytes plays an important role in binding and activation of sperm cells during the molecular events leading to fertilization. The genes coding for the three zona pellucida glycoproteins ZPA, ZPB, and ZPC of various species including mouse, dog, and human have been cloned and sequenced by several groups. However, it has remained a matter of debate as to whether the oocytes alone or in conjunction with the surrounding granulosa cells express and deposit these proteins to form the zona pellucida matrix. Addressing this unresolved issue, we assessed the expression and localization of all three zona pellucida proteins in ovaries of human, cynomolgus monkey and mice using immunohistochemical methods. In addition, oocyte-specific expression of ZPC from the primordial stage onward was confirmed by in situ hybridization. In sections of human ovaries, ZPA, ZPB, and ZPC proteins were immunohistochemically detected in the cytoplasms of primordial oocytes and during later stages of folliculogenesis in the zona pellucida matrices of oocytes. In sections fixed with formalin, a clear homogeneous ring was visible around the oocyte and no staining of granulosa cells was observed. In contrast, staining of ZP proteins was also observed between granulosa cells when Bouin's reagent had been used for tissue fixation. Thus, the original zona pellucida architecture was better preserved by formalin fixation. We further demonstrated that dissolution of the zona pellucida of isolated bovine oocytes occurred after they were exposed to Bouin's reagent. In summary, these results demonstrate that in mice, monkeys and humans, zona proteins are expressed and assembled exclusively by the oocyte and not by the granulosa cells. Previously observed results of ZP expression by an involvement of granulosa cells might therefore be the result of an improper fixation of the tissues leading to the disruption of the zona pellucida. Additionally this study highlights the importance of choosing the correct fixative for immunohistochemistry, not only for the usual reason of retaining antigenicity, but rather to retain the entire architectural structure.

**Keywords** Zona pellucida · Immunohistochemistry · In situ hybridization · Localization · Fixation · Human, *Macaca fascicularis*, Mouse (NMRI)

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## Introduction

Mammalian oocytes are surrounded by the zona pellucida, an extracellular matrix that is composed of three glycoproteins originally termed ZP1, ZP2 and ZP3 according to their apparent molecular weights on sodium dodecyl sulfate (SDS)-polyacrylamide gels (Bleil and Wassarman 1980). As the zona proteins are highly but variably glycosylated in different species, it was recently proposed to rank the zona proteins rather according to the length of their coding regions. This led to the use of a different nomenclature: ZPA, ZPB, and ZPC (Harris et al. 1994); in the mouse (and the human) ZP1 thus corresponds to ZPB, ZP2 to ZPA and ZP3 to ZPC.

The zona pellucida has a central role during the recognition and binding of spermatozoa and induces a cascade of biochemical events in sperm leading to the acrosome reaction (for review, see Wassarman 1988; Dean 1992). The zona pellucida is formed during follicular development in the ovary, persists at the time of fertilization within the oviduct, and surrounds the fertilized egg until implantation. Although the primary structures and biochemical properties of the ZP proteins from different species have been studied in detail, the precise site of synthesis of the human ZP proteins in particular remains a matter of debate. ZP proteins have been reported to be synthesized exclusively by the oocytes (Bousquet et al.

1981) or by both the oocytes and the surrounding granulosa cells (Hinsch et al. 1994; Lee and Dunbar 1993) in human ovaries. During oogenesis in mice all zona pellucida genes are expressed in a coordinated, oocyte-specific manner (Epifano et al. 1995).

The zona pellucida has been extensively characterized in the mouse (Wassarman 1988, 1990; Epifano and Dean 1994; Dean 1994). Here ZPC (ZP3) is responsible for species-specific binding of spermatozoa (Florman and Wassarman 1985) and for the induction of the acrosome reaction (Saling 1991), a prerequisite for fertilization. ZPA (ZP2) is thought to function as the secondary receptor (Bleil et al. 1988), whereas ZPB (ZP1) is believed to be a homodimeric cross-linker of ZPA and ZPC (Greve and Wassarman 1985).

Much of what has been learned about the structure and function of the zona pellucida components in the mouse does not necessarily apply to other mammals including humans. Experiments performed with porcine zona pellucida components, for example, have demonstrated that ZPB (pZP3α) might be responsible for sperm binding (Sacco et al. 1989; Yurewicz et al. 1993) rather than ZPC as shown in mice (Bleil and Wassarman 1980).

The potential use of recombinant zona pellucida proteins as antigens for immunocontraception has stimulated research in this field (Paterson and Aitken 1990; Talwar and Raghupathy 1995). Although fertility has been successfully reduced in rabbits and monkeys through immunization with various zona pellucida preparations (Paterson et al. 1992 and references therein), the immunocontraceptive effect was often accompanied by termination of ovarian cyclicity caused by depletion of recruited and, even more severe, of primordial follicles (Paterson et al. 1998). The cause of this ovarian pathology, especially the depletion of primordial follicles after immunization with recombinant ZPC, has remained elusive since ZPC mRNA and proteins were not detected in mouse (Philpott et al. 1987; Kimura et al. 1994) and marmoset monkey primordial follicles (Thillai Koothan et al. 1993). In addition, it has still not been fully resolved whether ZP expression is an oocyte-specific event or whether granulosa cells are involved in this process.

In this study, we used both immunohistochemical and in situ hybridization techniques to investigate the expression of zona proteins in human and other mammalian follicles. To do so we generated highly specific antisera raised against all three ZP proteins and analyzed the effect of different fixation techniques on the preservation of the zona pellucida architecture.

## Materials and methods

### Collection of native material

Human ovaries were obtained from regularly cycling premenopausal women (not older than 44 years) undergoing total abdominal hysterectomy and oophorectomy for non-ovarian indications, and were kindly provided by Dr. Artur Jakimiuk from the University School of Medicine in Lublin, Poland. Informed consent was obtained from all subjects participating in the study as approved by the Ethics Committee at the University School of Medicine in Lublin. Mammalian ovaries were also obtained from adult (90- to 120-day-old) NMRI mice (Naval Medical Research Institute, Bethesda, MD), cynomolgus monkeys (*Macaca fascicularis*; Affenzentrum, Münster, Germany) and pigs (local slaughterhouse).

### Protein chemistry

#### *Production of recombinant zona pellucida*

Recombinant human His-tagged proteins ZPA, ZPB and ZPC were expressed in Chinese hamster ovary (CHO) cells as already described (Harris et al. 1999). CHO supernatants were adjusted to 30 mM imidazole, 50 mM Na-Pi pH 7.9 and 0.05% 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propane sulfonate (CHAPS) and bound to a Ni-chelate-Sepharose. After washing in 30 mM imidazole, 50 mM Na-Pi, pH 7.9, 100 mM NaCl, 0.05% CHAPS, the proteins were eluted with a linear gradient of 0–300 mM imidazole in the same buffer and subsequently dialyzed in phosphate-buffered saline (PBS) supplemented with 0.05% CHAPS.

#### *Gel analysis*

Electrophoresis was carried out in 0.75 mm 12.5% SDS polyacrylamide gels as described (Laemmli 1970). The proteins were dissolved in sample buffer containing 0.1 M dithiothreitol (DTT). Determination of molecular weight was based on standard proteins (Pharmacia). Gels were stained for 15 min with 0.02% Coomassie brilliant blue G250 in 10% acetic acid and destained with 10% acetic acid.

#### *Western blot*

Proteins were transferred from SDS-PA gels to nitrocellulose sheets (BA 85; Schleicher & Schüll, Dassel, Germany) essentially as previously described (Towbin et al. 1979). The sheets were blocked for 1 h at 25°C containing 5% dry fat milk powder in PBS (Dulbecco and Vogt 1954), 0.05% Tween-20 and incubated (1.5 h, 25°C) with antisera (1:1000 in PBS, 0.05% Tween-20). The membranes were then washed thoroughly in PBS, 0.05% Tween before incubation (1.5 h at 25°C) with alkaline phosphatase-linked goat anti-rabbit IgG (Sigma, Deisenhofen, Germany). After intensive washing, reactive bands were visualized with 0.1 mg/ml nitroblue tetrazolium (NBT), 0.05 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100 mM TRIS [(hydroxymethyl) aminoethane]-HCl, pH 9.5, with 5 mM $MgCl_2$.

### Immunohistochemistry

#### *Production of antisera*

Since recombinant ZPA was not available at the beginning of this study, peptides were generated in order to induce ZPA-specific antibodies. Selection of two synthetic peptides from the corresponding human (Hu) ZPA sequence was based on the probability of their being antigenic as predicted by primary structure analysis using the Genetics Computer Group program package (GCG; Devereux et al. 1984), and the presence of highly conserved regions between species (see Table 1). The selected peptide sequences showed neither homology to ZPB nor ZPC sequences of other mammalian species.

Both peptides were generated on a HMP (4-hydroxymethylphenoxymethyl-copolystyrene-1% divinylbenzene) resin by automated solid-phase peptide synthesis (431A synthesizer; Applied Biosystems, Foster City, CA) using Fmoc chemistry. The peptides were cleaved off the resin (King et al. 1990) and purified by reversed-phase high-performance liquid chromatography (HPLC).

The structures were confirmed by sequencing and by mass spectrometry (MALDI-TOF). To enhance antigen presentation in vivo, peptides were coupled to keyhole limpet hemocyanin (KLH) (Reichlin 1980).

Rabbits were immunized either with a KLH-coupled mixture of two synthetic ZPA peptides, or with 100 µg purified recombinant huZPB or huZPC per injection (see Harris et al. 1999). The antigen was emulsified in Freund's complete (first injection) or incomplete (booster doses) adjuvant and injected subcutaneously every 3 weeks over a 12-week period. The antisera were generated at Eurogentec (Seraing, Belgium).

*Isolation and incubation of porcine oocytes in Bouin's solution*

Porcine oocytes with intact zonae pellucidae were recovered from ovaries by aspirating follicle fluid with a small syringe followed by several washing steps in PBS using a glass pipette. Finally the oocytes were placed in a drop of Bouin's fluid (saturated aqueous picric acid solution, 40% formaldehyde, glacial acetic acid; 70:25:5) and the zona pellucida was observed using a microscope (Axiophot, Zeiss, Oberkochen, Germany).

*Processing of ovaries for histological studies*

Freshly prepared ovaries were cut into halves and fixed at 4°C in 10% formalin (Lillie 1954) or Bouin's fluid (saturated aqueous picric acid solution, 40% formaldehyde, glacial acetic acid; 70:25:5). After 24 h of fixation, the tissue was washed in a graded ethanol series (70%, 95%, 100%) and embedded in paraffin wax. Tissue sections (2 µm) were placed on Superfrost Plus microscope slides (Menzel, Braunschweig, Germany), dried at 42°C for 24 h and stored.

*Immunohistological staining*

Tissue sections (2 µm) were deparaffinized, rehydrated, and incubated in 0.5% $H_2O_2$ in PBS in order to quench endogenous peroxidase activity. To avoid any non-specific reactivity of the antibodies, the sections were pretreated with 3% normal goat serum (diluted in PBS) for 1 h, and incubated with ZP antisera (dilution 1:500 or 1:1000 in PBS) overnight at 4°C. The sections were washed 3 times for 5 min each with PBS before the peroxidase-conjugated secondary antibody (goat anti-rabbit-POD, Sigma, Deisenhofen, Germany; dilution 1:1000 in PBS) was added for 1 h at 25°C. After a final wash in PBS, the immunoreactive sites were visualized with the peroxidase substrate 3,3'-diaminobenzidine (DAB; DAKO, Hamburg, Germany) or 3-amino-9-ethylcarbazol (AEC; DAKO, Hamburg, Germany). The sections were counterstained with hematoxylin and mounted either in Entellan Neu (Merck, Darmstadt) or Glycergel (DAKO, Hamburg, Germany). Preimmune sera were used in control experiments. Slides were observed under a Zeiss Axiophot microscope. Micrographs were taken using Ektochrome 320T film (Kodak).

PBS or Bouin's treated porcine oocytes were washed several times in PBS and placed overnight in ZPC antiserum (diluted 1:200 in PBS). After several washes in PBS the oocytes were placed in PBS, 0.05% Tween-20, and 5% goat serum for 20 min at room temperature. The oocytes were washed 3 times for 5 min each with PBS before the peroxidase-conjugated secondary antibody was added. The immunoreactive sites were visualized with the peroxidase substrate 3,3'-diaminobenzidine (DAB; DAKO, Hamburg, Germany).

In situ hybridization

*Probes and labeling*

Antisense and sense cRNA probes of HuZPC cDNA fragment (318 bp, position 749–1066; Chamberlin and Dean 1990), generated by enzymatic restriction of the full-length cDNA clone originally isolated by Harris et al. (1994), were cloned into pBluescript KS(+) vectors (Stratagene, La Jolla, CA). $^{35}$S-UTP and $^{35}$S-CTP (Amersham, Bucks., UK) were incorporated into the riboprobes according to the manufacturer's instructions (Boehringer, Mannheim, Germany) using T3 and T7 promoters. Riboprobes labeled with $^{35}$S were precipitated in 0.15 M sodium acetate and 75% ethanol, centrifuged at 14,000 rpm for 30 min, washed in 70% ethanol, and stored in 10 mM TRIS-HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM dithiothreitol (DTT). The size of transcribed riboprobes was verified by autoradiography following electrophoresis in 8% urea-polyacrylamide gels (Sambrook et al. 1989). The specific activity of $^{35}$S-riboprobes was $3\times10^6$ cpm/µl.

*In situ hybridization procedure*

Unstained tissue sections were deparaffinized in xylene, rehydrated through a descending ethanol series, washed in PBS, and incubated for 20 min in 0.2 N HCl, and then for a further 20 min in 0.3% Triton-X 100 at room temperature. Sections were treated with RNase-free proteinase K (5 µg/ml), washed twice with 0.2% glycine for 5 min, and acetylated with 0.25% (v:v) acetic anhydride (Sigma Chemical Co., St. Louis, MO) in 0.1 M triethanolamine buffer (pH 8.0; Sigma Chemie, Deisenhofen, Germany) at room temperature for 10 min. After washing with PBS, slides were prehybridized for 2 h at 62°C in a single-strength Denhardt's solution consisting of 0.02% Ficoll, 0.02% bovine serum albumin (BSA); 0.02% polyvinylpyrrolidone; containing 50% [v:v] formamide, 0.3 M NaCl, 20 mM TRIS-HCl (pH 8.0), 1 mM EDTA, 10% dextran sulfate, 500 µg/ml yeast tRNA and 100 mM dithiothreitol (DTT).

Slides were incubated in a humidified chamber at 62°C for 24 h in hybridization solution containing $^{35}$S-labeled cRNA probe ($2\times10^6$ cpm/slide). Posthybridization washes included 5 min in 5×SSC (1×SSC consists of 0.15 M sodium chloride, 0.015 M sodium citrate) containing 10 mM DTT for 5 min at room temperature, treatment with 50 µg ml RNase A (Merck, Darmstadt, Germany) in RNase A-buffer (10 mM TRIS-HCl, 1 mM EDTA, 0.5 M NaCl pH 8.0) at 37°C for 30 min; and were then washed for 10 min in RNase A-buffer at room temperature and for 1 h in 2×SSC/50% formamide plus 1 mM DTT at 60°C. The slides were rinsed at 60°C in 2×SSC, then twice in 0.1×SSC for 15 min each, dehydrated through a graded ethanol series, air dried, and exposed overnight to Biomax MR film (Kodak, Rochester, NY) in order to roughly estimate the time required for exposure before using the photographic emulsion. The slides were then coated with photographic emulsion (LM-1-RPN, Amersham, Bucks., UK) and stored in complete darkness at 4°C for 2–6 weeks. The emulsion was developed (Kodak D-l9, Paris, France), fixed in Kodafix (Kodak, Paris, France), and sections were counterstained with Eosin Y (Sigma, Deisenhofen, Germany). After dehydrating sections through an ascending series of ethanol, they were cleared in xylene and coverslips were mounted with Entellan Neu (Merck, Darmstadt, Germany).

## Results

### Characterization of ZP sera

Polyclonal antibodies to ZPA were generated by immunizing rabbits with a combination of two synthetic peptides corresponding to ZPA sequences fully conserved across different species (see Fig. 1). Polyclonal antibodies reacting with ZPB or ZPC were generated by immunization of rabbits with highly purified recombinant human ZPB or ZPC proteins, respectively.

| Peptide 1 | | Species | | Peptide 2 |
|---|---|---|---|---|
| CGTRQKFEGDKVIYE | | Mouse | | SRNDPNIKLVLDDC |
| 424 **CGTRYKFEDDKVVYE** 438 | | Human | 532 | **NRDDPNIKLVLDDC** 545 |
| CGTRHKFEDEKVIYE | | Rabbit | | NRNDPNIKLALDDC |

**Fig. 1** Synthetic peptide amino acid sequences (*bold*) from the HuZPA sequence used for immunization in comparison with corresponding ZPA sequences of two other mammalian species. Amino acid sequence deduced from human (Liang and Dean 1993), mouse (Liang et al. 1990) and rabbit (Lee et al. 1993b) cDNA

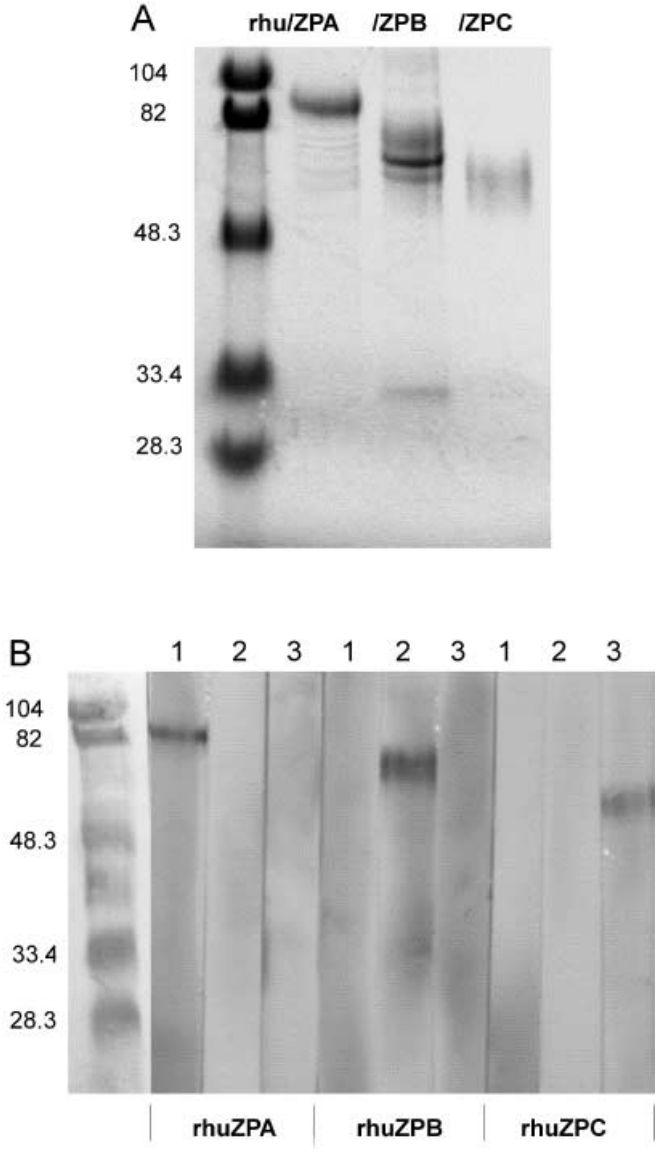



**Fig. 2** Purified recombinant human His-tagged ZP proteins separated by SDS-PAGE and stained with Coomassie brilliant blue G250 (2 µg protein/lane; **A**) or analyzed by protein immunoblotting (0.1 µg protein/lane; **B**). Nitrocellulose strips onto which all ZP variants had been blotted were incubated with anti-ZPA (*1*), anti ZPB (*2*) or anti-ZPC (*3*); antibody dilutions were 1:1000

The specificity and selectivity of the ZP antisera used in this study were evaluated on immunoblots of recombinant human ZP proteins separated by SDS-PAGE (Fig. 2). As expected, all ZP antisera reacted specifically with the corresponding zona proteins; they did not cross-react.

## Localization of zona proteins in the ovary

Formalin-fixed and paraffin-embedded sections of human, cynomolgus monkey, and mouse ovaries were used to assess zona pellucida protein expression during different stages of folliculogenesis. Using the specific antibodies described above, staining of all three ZP proteins was observed in the ovaries of the aforementioned species. The presence of all three proteins was confined to the adjacent extracellular area around the oocyte with virtually no staining in or between granulosa cells. (Figure 3 shows this for cynomolgus ovaries.) The peptide-specific antibody recognizing ZPA (Fig. 3b) caused a weaker signal than that observed for ZPB and ZPC antisera (Fig. 3d, f, respectively).

In human oocytes, all ZP proteins could be detected from primordial stages onward (Fig. 4 shows this representative for ZPC). In primary and more developed follicles, the observed staining displayed the shape of a distinct ring around the oocyte representing the glycoprotein matrix of the zona pellucida (Fig. 4c). In resting oocytes of primordial follicles, expression of zona proteins was detectable in the cytoplasm (Fig. 4a).

There is no agreement in the literature as to whether the oocyte, the granulosa cells or both are involved in the synthesis of zona proteins. In reviewing the evidence for either alternative, we came to the conclusion that the reagent used for fixation of the tissue could be responsible for this discrepancy. Since it is well established that in the mouse the oocytes synthesize the proteins that constitute the zona pellucida (Epifano et al. 1995), we chose mice to further investigate the effects of fixation on the precise localization of zona pellucida proteins. When formalin was used for the fixation of the ovaries and ZPC antisera for immunohistochemistry, zona-pellucida-specific staining was confined to a ring encircling the oocyte without protrusion into the granulosa cell layer (Fig. 5a, b, e). In contrast, when Bouin's reagent was used for fixation, the zona pellucida structure was almost completely destroyed and staining occurred between the first and second layers of the surrounding granulosa cells (Fig. 5c, d, f).

This effect was not only restricted to mouse ovaries, as we were able to reproduce this result in ovarian sections from both rat and pig (data not shown).

## Solubilization of zona pellucida in Bouin's solution

Isolated porcine oocytes surrounded by an intact zona pellucida matrix were transferred from PBS into Bouin's

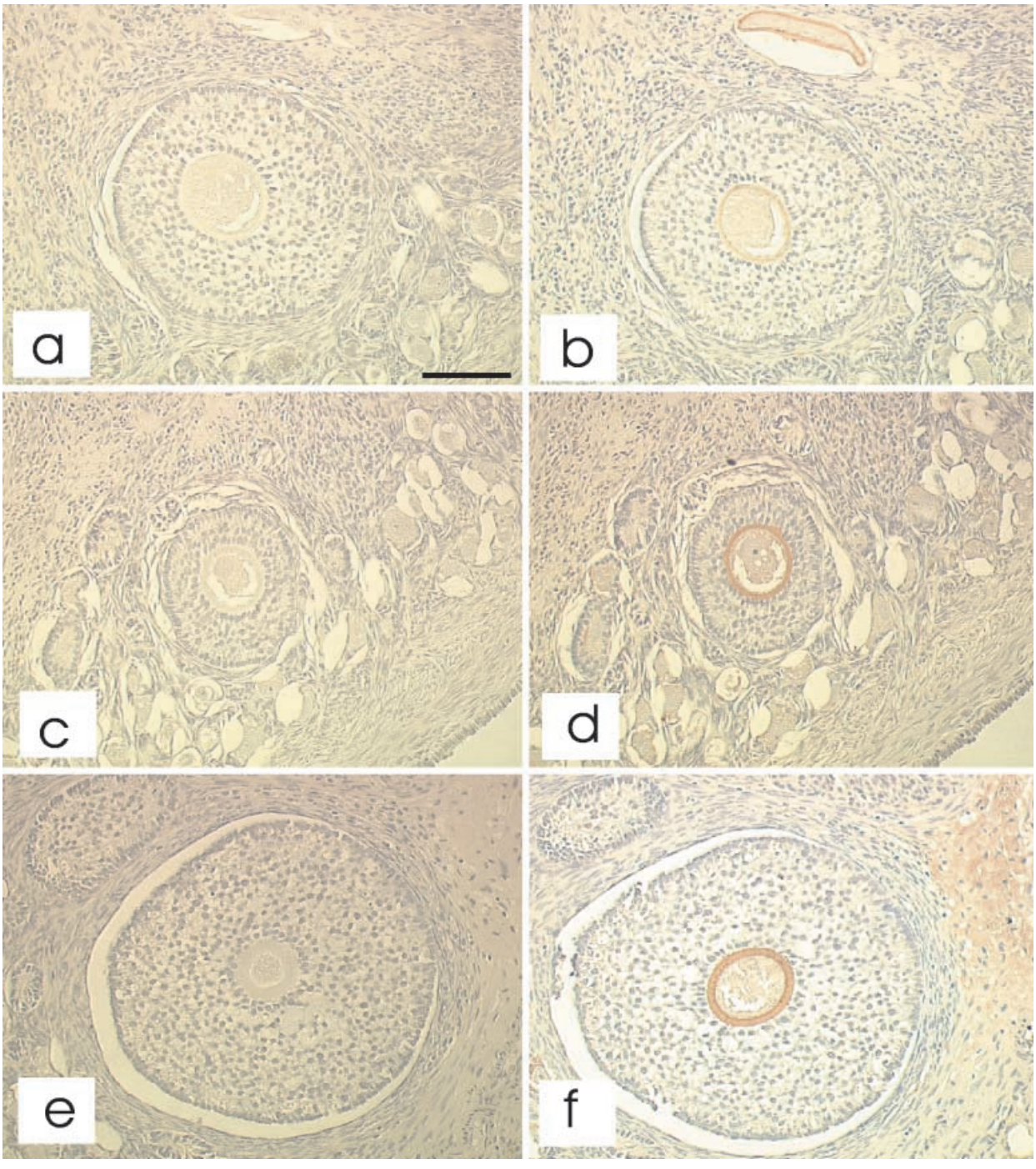



**Fig. 3a–f** Immunocytochemical localization of ZPA, ZPB and ZPC in follicles of cynomolgus monkey ovaries. The tissue was fixed in formalin and immunostained with anti-ZPA **(b)**, anti-ZPB **(d)** or anti-ZPC **(f)**. Subsequent sections were stained with corresponding preimmune sera **(a, c, e)**. *Bars* 100 µm

solution. Within seconds the opaque zona ring around the oocytes disappeared (Fig. 6a, b). Immunohistochemical staining of these oocytes using a ZPC antibody revealed that the ZPC-containing layer that remained on the oocyte surface of Bouin's-treated oocytes (Fig. 6d) was thinner than that on the PBS-treated controls (Fig. 6c). Loss of the zona is evidenced not only by higher light transparency caused by a weaker staining intensity with ZPC antibody, but also by the loss of a smoothly shaped form (Fig. 6a, c).

## In situ hybridization of ZPC mRNA

In order to localize ZPC mRNA transcripts, in human ovaries, organs were fixed, sectioned, and hybridized in situ with sense and antisense $^{35}$S-labeled cRNA probes (see "Materials and methods").

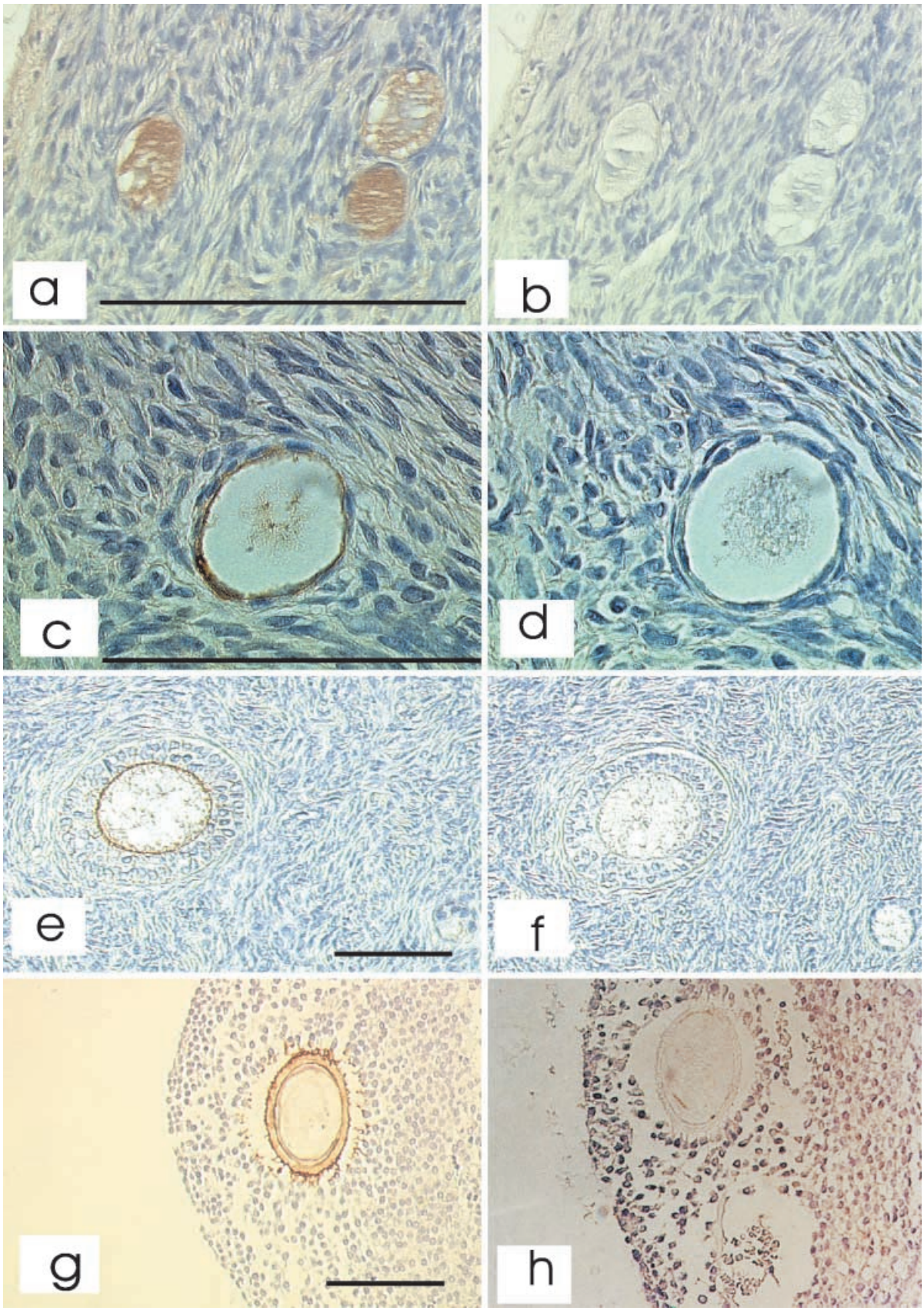



**Fig. 4** Immunocytochemical localization of ZPC proteins in the human ovary showing follicles in different maturation stages from primordial **(a)**, primary **(c)**, secondary **(e)** and antral follicles **(g),** respectively. Subsequent sections were stained with corresponding preimmune sera **(b, d, f, h)**. *Bars* 100 µm

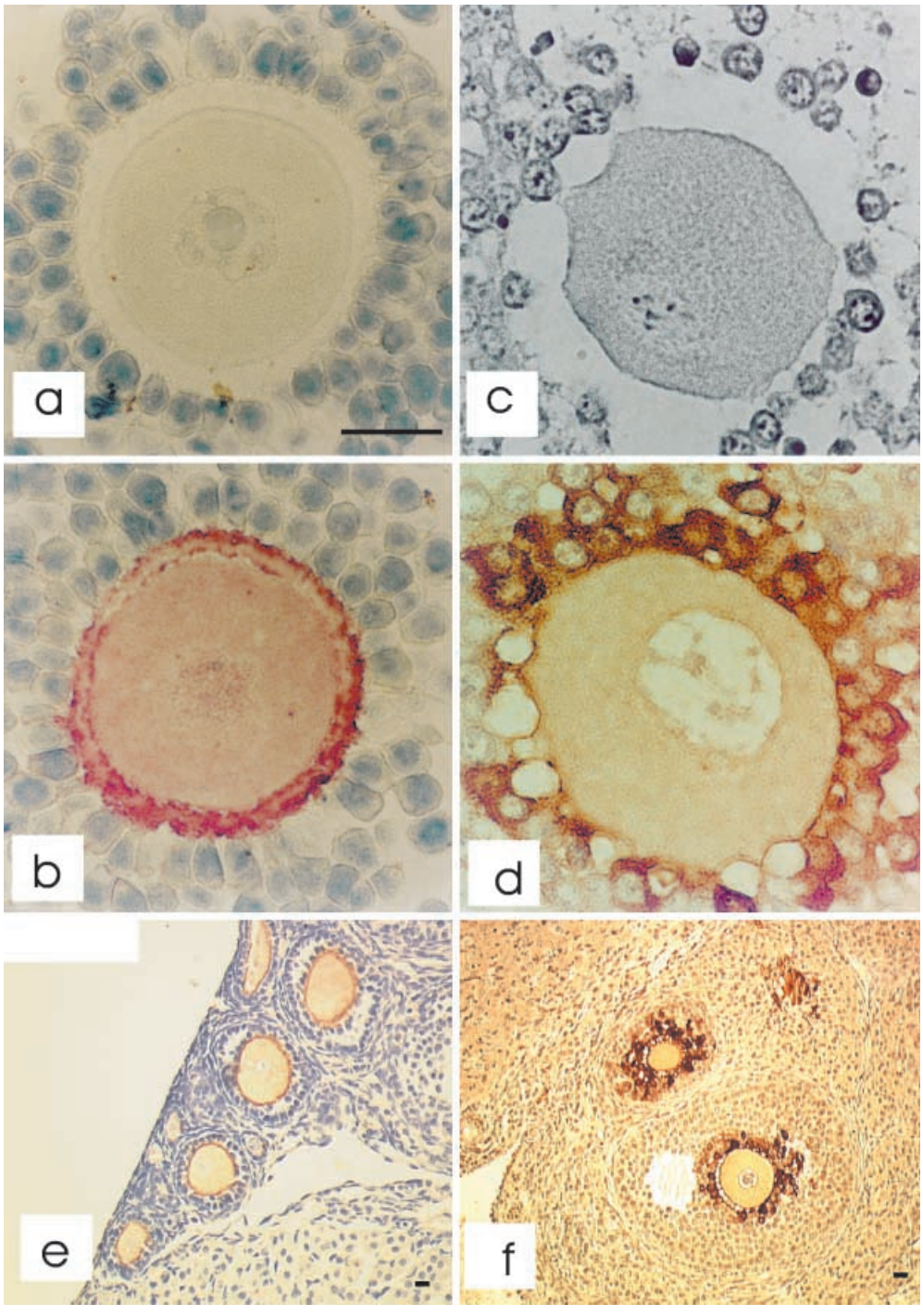



**Fig. 5** Immunocytochemical localization of ZPC in follicles of the mouse ovary fixed either in formalin **(a, b, e)** or Bouin's fluid **(c, d, f)**. Sections were immunostained with anti-ZPC **(b, d, e, f)** or the corresponding preimmune serum **(a, c)**. Immune complexes were detected with AEC substrate for **b, e,** and **f** and DAB substrate for **d**. ×1000. *Bars* 20 µm

Small groups of primordial follicles were visible in the ovarian cortex (Fig. 7). They consisted of an oocyte surrounded by a single layer of squamous follicle cells resting in contact with a basal lamina. Human ZPC mRNA transcripts were detected in the cytoplasm of resting oocytes of primordial follicles (Fig. 7a, b), as well as in growing oocytes up to the stage of secondary follicle (Fig. 7c). Corresponding control sections treated with the sense probe were not stained (Fig. 7d, e).

## Discussion

We have generated polyclonal antisera which are highly specific to each of the human zona pellucida proteins (Fig. 2). However, they do cross-react with the homologous proteins of other mammalian species, e.g., cynomolgus monkey, mice (shown here) but also with rat and rabbit ZP proteins (data not shown). In this study, antisera have been used to monitor the expression of zona proteins during follicular development in mice, cynomolgus

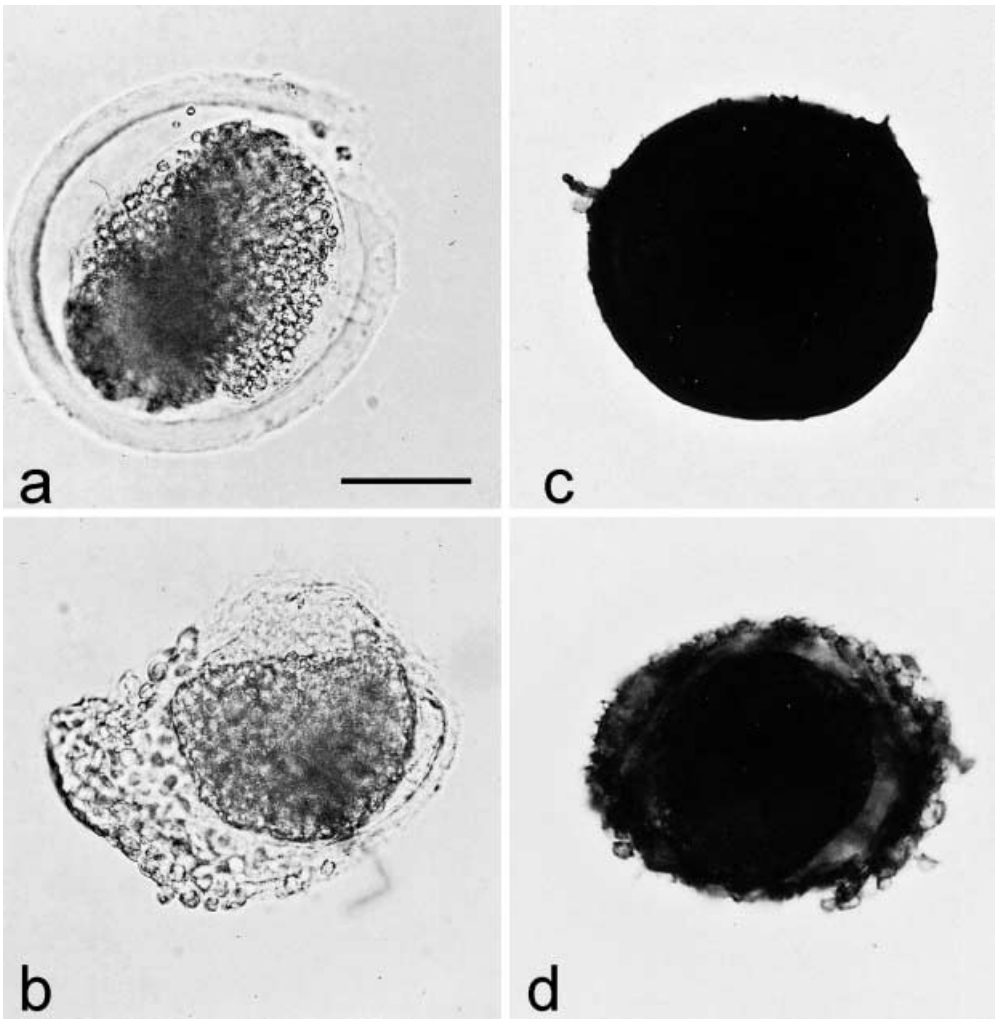



**Fig. 6** Isolated porcine oocytes incubated in PBS **(a)** or Bouin's **(b)**. Oocytes were stained with anti-ZPC serum **(c, d)**. *Bars* 20 µm

monkeys and humans. In addition, we carried out experiments to clarify whether the zona proteins are exclusively expressed and deposited by the oocytes or whether granulosa cells are also involved in this process.

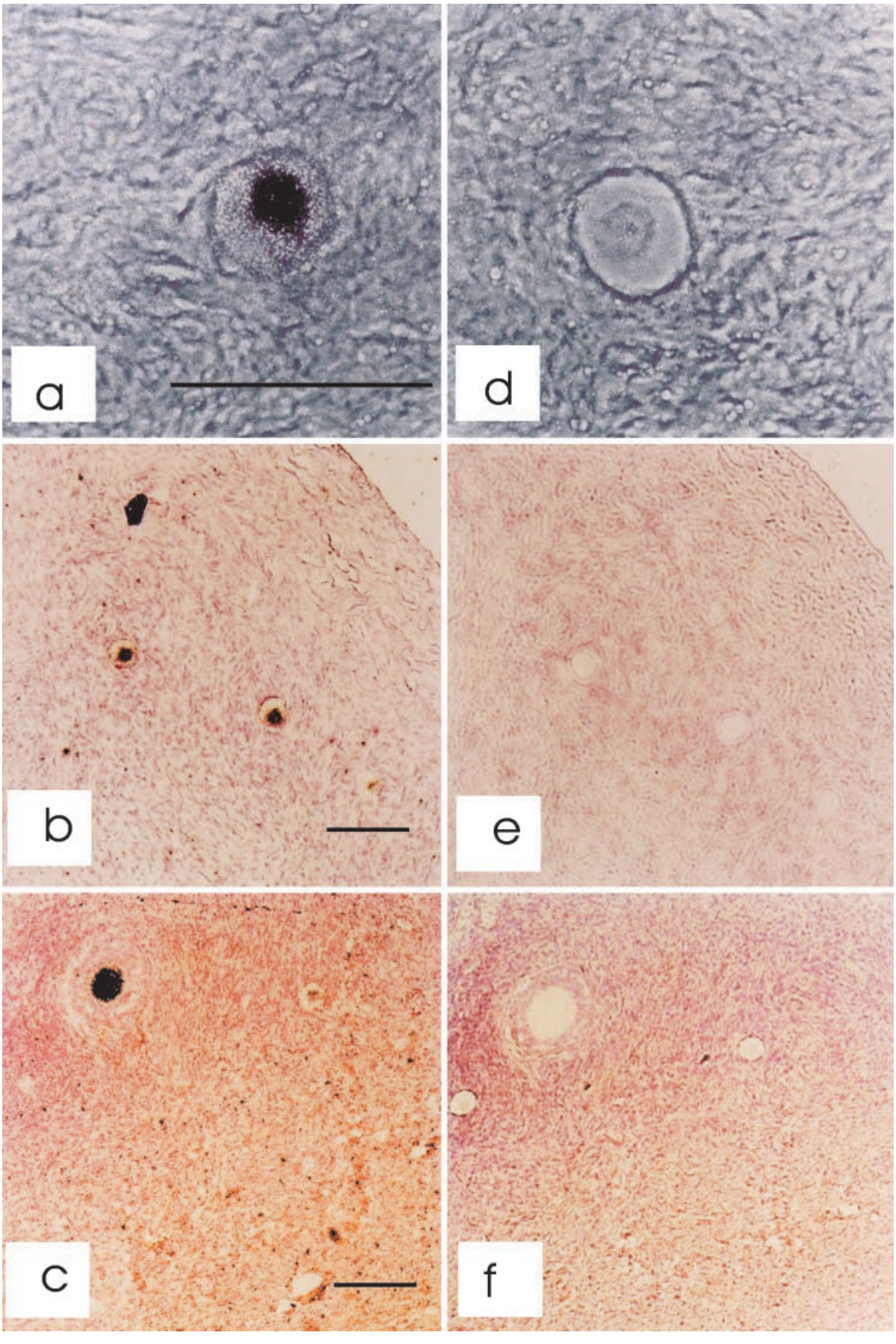
In resting human oocytes, ZPA, ZPB and ZPC (see Fig. 4a) could not be detected around the oocytes, indicating that a zona pellucida had not yet been formed. This is in agreement with data obtained in mice where no zona pellucida was found around primordial oocytes (Wassarman 1983). Instead, staining for zona pellucida proteins was found in the cytoplasm at this stage, indicating that zona protein expression had already been initiated but their secretion and assembly into the zona matrix had not yet begun.

In the case of ZPC, we were able to confirm these data on sections of human ovaries by in situ hybridization, showing that mRNA transcripts for ZPC already exist at the early, resting stage of follicular development.

Our findings are in agreement with data obtained in mouse oocytes in vitro, where transcription/translation was found to be initiated in primordial oocytes (Epifano et al. 1995). The formation of the zona pellucida was first detectable at the primary follicle stage (see Fig. 4c) using a ZPC-specific antibody.

In our sections staining was strictly confined to the zona pellucida, exhibiting a circular shape surrounding the oocyte. There is an ongoing debate as to whether zona proteins are exclusively synthesized exclusively by the oocytes, or whether granulosa cells in some species contribute to the formation of the zona pellucida. In the literature both concepts are supported by immunohistochemical data. In support of the latter, it has been speculated that the rate of protein synthesis by oocytes is too low to account for its own mature weight in addition to that of the zona (Schultz et al. 1979a, 1979b). It was argued that the oocyte does not have enough time after its recruitment to synthesize the zona since it makes up 20% of total protein mass in oocytes (Sacco 1990).

**Fig. 7** In situ hybridization of mRNA using cRNA probes of human ZPC in human ovaries; 2-µm paraffin sections. **a–c** Antisense probes; **d–f** sense probes. Sections **a** and **d** were not counterstained with eosin Y; sections **b, c, d** and **f** were counterstained with eosin Y. *Bars* 100 µm ▶


a
d
b
e
c
f

Although different localizations of the zona proteins might reflect differences among mammalian species, we thought it possible that variations in the histochemical methods used to prepare the tissue before sectioning might be responsible for the divergent results. Thus, when Bouin's fluid was used as the fixative, zona protein staining was detected between the granulosa cells surrounding the zona pellucida matrix as well as in the zona pellucida matrix in porcine (Sinowatz et al. 1995) and rabbit ovaries (Prasad et al. 1996). Accordingly, we saw the same staining pattern between granulosa cells as the two aforementioned groups when we used this fixation technique (see Fig. 5d, e).

In contrast, when paraformaldehyde (Takagi et al. 1989) or, as described in this study, formalin-fixed sections were used, staining was confined to the zona pellucida (see Figs. 3b, d, f, 4c, e, g, 5b, e).

In this context we would like to mention a publication (Grootenhuis et al. 1996) that evaluated different fixation techniques. Using Bouin's fixative the morphology of the ovary was optimal, although the immunocytochemical staining of the ZP was possible only in recruited follicles. This observation might be due to the fact that small zonae pellucidae structures in earlier follicle stages had been solubilized by Bouin's fixative. The authors demonstrated that using various paraformaldehyde fixation techniques the ZP was thicker than that observed with Bouin's fixative. Based on this observation, the authors concluded that ZP was probably better preserved in its original status by the latter fixation method.

Therefore we tested the hypothesis that the observed differences in ZP staining are due to the method of fixation used. Staining in formalin-fixed sections was indeed confined to the zona, while after Bouin's fixation we detected staining between the granulosa cells surrounding the zona pellucida in all species examined.

These artifacts are likely due to partial disintegration of the zona pellucida caused by the highly acidic Bouin's fixative as has been described for the preparation of soluble zona pellucida with other acidic buffers (Franken et al. 1996). Subsequent diffusion of dissolved zona constituents would result in their immunohistochemical detection in the granulosa cell layers adjacent to the oocyte. This interpretation is corroborated by an in vitro experiment in which Bouin's solution was able to solubilize intact ZP matrices of isolated porcine oocytes within seconds (Fig. 7).

To substantiate these findings, we carried out in situ hybridization with labeled ZPC-specific cRNA probes. hZPC mRNA transcripts were specifically found in the oocytes and no staining was observed in granulosa cells of primary follicles. Previously, it had been described that in the early stages of follicular development in pigs the ZPC mRNA is predominantly expressed in the oocyte whereas from secondary follicle stages onward granulosa cells were also found to synthesize ZPC mRNA (Koelle et al. 1996). Our data do not support this observation: we were unable to detect immunohistochemical signals in or around granulosa cells of the cumuli oophori neither in humans nor in the other species examined (Figs. 4, 5, 6). Others have detected ZPA mRNA transcripts in mice by in situ hybridization in resting oocytes, whereas ZPB and ZPC transcripts were detected only after the oocytes had begun to grow (Epifano et al. 1995). In contrast, we were able to detect transcripts of ZPC in human primordial follicles as well.

In conclusion, our findings provide a rationale for the divergent reports concerning the expression and deposition of zona proteins. We have demonstrated that formalin fixation preserves the molecular structural integrity of the zona pellucida as a well-defined halo around the oocyte, whereas Bouin's fixative does not. These observations should be taken into account in future studies of zona pellucida structure.

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