Human Reproduction, Vol.28, No.3 pp. 718–728, 2013
Advanced Access publication on January 4, 2013 doi:10.1093/humrep/des423

human reproduction

ORIGINAL ARTICLE *Reproductive biology*

The human is an exception to the evolutionarily-conserved phenomenon of pre-fertilization zona pellucida resistance to proteolysis induced by oviductal fluid

I. Mondéjar[1,2], M. Avilés[3], and P. Coy[1,2,*]

[1]Department of Physiology, Veterinary Faculty, University of Murcia, Campus Mare Nostrum, Espinardo 30100, Murcia, Spain [2]Instituto Murciano de Investigación Biosanitaria (IMIB), Murcia, Spain [3]Department of Cell Biology and Histology, Faculty of Medicine, University of Murcia, Campus Mare Nostrum, Espinardo 30100, Murcia, Spain

*Correspondence address. Email: pcoy@um.es

Submitted on July 17, 2012; resubmitted on November 6, 2012; accepted on November 9, 2012

STUDY QUESTION: Is zona pellucida (ZP) resistance to proteolysis, induced by oviductal fluid (OF), a mechanism common to species other than the pig and cow?

SUMMARY ANSWER: ZP resistance to proteolysis induced by OF was observed in the mouse, rat, hamster, rabbit, sheep, goat, pig and cow, but not in humans.

WHAT IS KNOWN ALREADY: Oviductal ZP resistance to proteolysis occurs in the pig and cow where it influences the incidence of fertilization and polyspermy. The effect is observed after incubation of ZP in OFs from pig (pOF), cow (cOF), rabbit (rOF) and sheep (sOF).

STUDY DESIGN, SIZE, DURATION: Oocytes from nine different species, including ungulates, rodents, lagomorphs and primates were incubated in rOF, sOF, gOF, cOF, pOF and human oviductal fluid (hOF). ZP digestion times for the matured oocytes of these nine species, without any treatment or incubated in 5 (mouse, rat, hamster, rabbit, cow, ewe and goat) or 6 (pig and humans) of the OFs collected were compared using three replicates per treatment and at least three oocytes per replicate.

MATERIALS, SETTING, METHODS: *In vivo* matured oocytes from rat, hamster, mouse, rabbit and humans, *in vitro* matured oocytes from cow, goat, ewe and pig and rOF, cOF, gOF, sOF, pOF and human (hOF) were collected and processed for the study. Oocytes from each species were incubated in the different OFs for 30 min. The resistance of the ZP of the oocytes to enzymatic digestion in a pronase solution (0.5% in PBS) was measured and registered as ZP digestion time.

MAIN RESULTS AND THE ROLE OF CHANCE: rOF increased ZP resistance to proteolytic digestion in the range of between 96 and 720 h for any of the species tested, whereas the corresponding increase in human ZP was only 1 min. OFs from the remaining species also had a significant effect, with variations among the cross-species experiments ($P < 0.05$). hOF, which was only tested on human and porcine oocytes, had no effect on ZP chemical hardening. Measurements of ZP digestion times are not of extreme accuracy and errors of a few seconds can be assumed in the experimental data. However, when differences are in the range of hours among treatments, variations measured in seconds do not alter the robustness of the findings.

LIMITATIONS, REASONS FOR CAUTION: Human oocytes and OF were of limited access, compared with oocytes from species collected in slaughterhouses. OFs from mouse, rat and hamster were not tested due to the small size of the genital tract in these species and the small volume of fluid available.

WIDER IMPLICATIONS OF THE FINDINGS: Since oviductal modification of ZP resistance to proteolytic digestion has been demonstrated to influence fertilization and this pre-fertilization mechanism is considered to contribute to the control of polyspermy, the apparent absence of this mechanism in humans suggests that the regulation of polyspermy depends mainly on other mechanisms, most probably of cortical granule origin. Investigation into a possible relationship between the lack of oviductal ZP hardening in human oocytes and the existence of tubal ectopic pregnancies in this species is proposed.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the Spanish Ministry of Science and Innovation and FEDER, Grant AGL2009-12512-C02-01-02. The authors declare no competing interest.

Key words: zona pellucida / hardening / oviductal fluid / fertilization / ectopic pregnancy

Introduction

In animals with internal fertilization, the oviduct provides the environment necessary for gamete transport and final maturation, fertilization and early embryonic development. It is well known that the sperm of many species need to spend some time in the oviductal reservoir to be capacitated, an essential requirement for their arrival at the site of fertilization and for penetrating the oocyte envelopes (Austin, 1951; Yanagimachi, 1994; reviewed by Suarez, 2008; Burkitt *et al.*, 2012). Oocytes also undergo modifications while they travel through the oviduct to the site of fertilization in the ampulla. One of these modifications at the zona pellucida (ZP) level involves an increase in resistance to digestion by proteolytic enzymes (enzymatic hardening of the zona), which, in turn, has been demonstrated to influence fertilization in the pig and cow: exposure of oocytes to oviductal fluid (OF) increases the incidence of monospermy after IVF by reducing sperm–ZP binding (Coy *et al.*, 2008). These changes, which could be considered as pre-fertilization modifications or oviductal maturation of the ZP, have not yet been studied in many species (Coy and Avilés, 2010).

The effect of enzymatic ZP hardening produced by contact with oviductal secretions prior to fertilization in these two species differs from the hardening that occurs as a result of the release of the cortical granule contents after fertilization in rodents (Gulyas and Yuan, 1985); in the humans, this post-fertilization enzymatic hardening remains controversial, while in the pig and cow it simply does not occur, at least not in *in vitro* fertilized oocytes (Coy and Avilés, 2010). In addition, this enzymatic hardening is also independent of, and not related to, the physical hardening described after fertilization in the cow, where it modifies the biomechanical properties of the ZP, inducing changes in the Young modulus and several elastic parameters (Boccaccio *et al.*, 2012).

The above-mentioned enzymatic ZP hardening before fertilization has been observed in the cow and pig oocytes (i) retrieved directly from the oviduct (Katska *et al.*, 1989; Kolbe and Holtz, 2005), (ii) after treatment with OF (Coy *et al.*, 2008) and (iii) when placed inside the isolated oviducts maintained in *in vitro* conditions. These effects were maintained when oocytes (cow) were placed in homologous (Smorag and Katska, 1988; Katska *et al.*, 1999) or heterologous (cow, sheep and rabbit) oviducts (Katska *et al.*, 1988, 1989) and so this process seems not to be species specific, at least among ruminants and rabbits.

OF is a complex mixture of components produced by oviductal epithelial cells and plasma transudate. At least two components of the OF, oviduct-specific glycoprotein (OVGP1) and heparin-like glycosaminoglycans, have been implicated in the pre-fertilization ZP hardening in the cow and pig (Coy *et al.*, 2008). Since both components are common to multiple species, we hypothesized that the OF from any species could induce ZP hardening in any other species. If so, we would be dealing with an evolutionarily conserved mechanism that influences gamete interactions. Since the treatment of oocytes with OF and with host oviducts has been shown to improve embryo development and quality (Lloyd *et al.*, 2009; reviewed by Rizos *et al.*, 2010) and to affect sperm motility (Coy *et al.*, 2010), and because the availability of OF from slaughterhouses is almost unlimited, the demonstration of our hypothesis could contribute to the development of a useful and common tool for improving *in vitro* embryo production in different species.

The aim of this study was to investigate whether OF from six different species has the capacity to modify the ZP of nine different species in a homologous and heterologous manner.

Materials and Methods

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Quimica SA (Madrid, Spain).

Ethical approvals

The experimental procedures for the use of animals and for the collection and use of human OF and oocytes were approved by the Ethical Committee of the University of Murcia and by the Ethical Committee for Clinical Research of the Reina Sofia University Hospital in Murcia, Spain, according to Spanish Law 14/2006 concerning human-assisted reproductive technologies (ARTs). All procedures were performed according to the Declaration of Helsinki for Medical Research involving Human Subjects and also to the Guiding Principles for the Care and Use of Animals (DHEW Publication, NIH, 80-23).

Collection of *in vivo* matured oocytes (rabbit, mouse, rat and hamster)

Laboratory animals used in this experiment were obtained from the animal house (eight adult females, 3–4 months old, of mouse, rat and hamster) and the Department of Pharmacology (five adult female rabbits, 1–2 years old) both of the University of Murcia. Rodents and rabbits were maintained under constant conditions of light and darkness, at room temperature, with free access to pelleted food and water. Each group of animals was injected (i.m. in rabbits and subcutaneous in rodents) with eCG (equine chorionic gonadotrophin, Folligon, Intervet International B.V. Mexico) and 48 h later with hCG (human chorionic gonadotrophin, Veterin corion, Divasa Farmavic, Spain). Equal doses of eCG and hCG were used as follows: 15 IU for rat, 25 IU for hamster, 5 IU for mouse and 40 IU for rabbits. After 17 h of hCG stimulation, rats were sacrificed and the ovulated oocytes were obtained from the oviductal ampulla. This approach was favored because *OVGP1* in this species is a pseudogene (rat oviduct lacks this protein) and, theoretically, there should be no ZP hardening in the oviduct. In rabbit, mouse and hamster the oocytes were obtained from the ovaries by slicing after sacrifice 12, 20 and 14 h after application of hCG, respectively. Cumulus–oocyte complexes (COCs) were collected in Dulbecco's PBS (DPBS) supplemented with 1 mg/ml polyvinyl alcohol (PVA) and 0.005 mg/ml red phenol under a temperature-controlled stereomicroscope and washed twice.

Human ZP and oocyte collection

Human oocytes (immature, ICSI failures and fragmented) were obtained from infertility clinics (IVI-Murcia and Tahe Fertility) with the consent of the donor and partners under the current regulations for the use of this material in Spain. Oocytes were transported to the laboratory on the same day or the day after follicular aspiration in the Global® Total® w/HEPES medium (LifeGlobal®, LGTH-250, Canada) and analyzed or used to collect ZPs immediately after transportation. ZPs were separated from the oocyte after introduction into bi-distilled H_2O, collected with a glass heat-thinned pipette and stored at −80°C until use.

Collection and *in vitro* maturation of oocytes (pig, cow, sheep and goat)

Pig

The medium used for pig oocyte maturation was NCSU-37, prepared in our laboratory as described previously (Coy *et al.*, 2002). Within 30 min of slaughter, ovaries from gilts were transported to the laboratory in saline containing 100 μg/ml kanamycin sulfate at 38°C, washed once in 0.04% cetrimide solution and twice in saline. Pig COCs were collected from antral follicles (3–6 mm diameter), washed twice with DPBS supplemented with 1 mg/ml PVA and 0.005 mg/ml red phenol, and twice more in maturation medium previously equilibrated for a minimum of 3 h at 38.5°C under 5% CO_2 in air. Only COCs with a complete and dense cumulus oophorus were used for the experiments. Groups of 50 COCs were cultured in 500 μl maturation medium for 22 h at 38.5°C under 5% CO_2 in air. After culture, the oocytes were washed twice in fresh maturation medium lacking dibutyryl cAMP, eCG and hCG, and cultured for an additional 20–22 h (Funahashi *et al.*, 1997).

Cow, sheep and goat

Cumulus–oocyte complexes were collected by aspiration from non-atretic follicles (2–6 mm diameter) from ovaries obtained from the slaughterhouse. All the ovaries came from animals between 14 and 20 months old. COCs were then washed twice in the TCM-199 medium with Hank's salts, 10.0 mM HEPES, 2% fetal bovine serum (FBS), 2.0 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin, and once in maturation medium previously equilibrated for 4 h at 38.5 °C under 5% CO_2 in air. Groups of 50 COCs were cultured in 500 μl maturation medium for 24 h at 38.5°C under 5% CO_2 in air.

Collection of OF

OFs from mouse, rat and hamster could not be collected due to the small body size of these species and the need to sacrifice them for the unique purpose of this study. Bovine, porcine, ovine and caprine oviducts were obtained from the slaughterhouses and transported to the laboratory at 4°C in saline. After that, specimens were classified as described previously (Carrasco *et al.*, 2008a,b) and only oviducts from animals in the late follicular phase of the estrous cycle were used. Oviducts from superovulated rabbits were obtained after the sacrifice of animals. The oviducts from each species were transferred to Petri dishes placed on ice, dissected and washed twice in saline solution. OFs from cow (cOF), pig (pOF), sheep (sOF), goat (gOF) and rabbit (rOF) were collected by aspiration with an automatic pipette using a tip regulated for a 200 μl volume and protease inhibitor (Complete Mini, EDTA-free, Ref.11 836 170 001, Roche, Spain) was added (1 μl 10× solution/10 μl OF). OF were centrifuged at 7000*g* for 10 min at 4°C to remove cellular debris and the supernatants were stored at −80°C. Samples were pooled, creating a stock of OF from each of the species, which was then stored at −80°C as aliquots (20 μl).The human OF (hOF) was obtained from the Reina Sofia University General Hospital from a 43-year-old woman (in the secretory phase of the menstrual cycle) who underwent hysterectomy due to the presence of a myoma; the signed consent of the patient was obtained. After salpingectomy, the oviducts, which were of healthy appearance, were cleaned of external residual blood with sterile gauzes and the OF was collected by aspiration.

Treatment of oocytes and ZPs with OF

Preliminary experiments revealed that the effect of OF on ZP resistance to pronase was independent of the presence or absence of cumulus cells (Coy *et al.*, 2008). Therefore, in all the experiments the cumulus cells were removed to facilitate oocyte manipulation and clear observation of the ZP. Oocytes from all species and human ZPs were incubated in undiluted OF (one oocyte or ZP per microliter of fluid) of each species and covered with mineral oil for 30 min at 38.5°C under 5% CO_2 in air. A control group of oocytes from each species was used in all the experiments; these groups were incubated at the same time in DPBS (one oocyte or ZP per microliter of DPBS) or in the LGTH medium for human oocytes covered with mineral oil on a hotplate at 38.5°C.

Assessment of ZP solubility

After incubation in OF, the oocytes or isolated ZPs were washed quickly in DPBS and transferred to 50 μl drops of 0.5% (wt/vol) pronase solution in DPBS to assess ZP digestion times. The dissolution time of the ZP of each oocyte was registered as the time when the zona was no longer visible under an inverted microscope equipped with a warm plate at 37°C at ×200 magnification. This time was referred to as 'ZP digestion time'. The experimental design of the study is explained in Fig. 1 and a video clip showing the different response to pronase between one control oocyte and one oocyte incubated in OF is available as Supplementary data, File S1.

Statistical analysis

Data are presented as the mean ± SEM. The variables in all of the experiments were analyzed by one-way ANOVA (ZP digestion time). When ANOVA revealed a significant effect, values were compared by the Tukey *post hoc* test. A P value of <0.05 was taken to denote statistical significance.

Results

Effect of six OFs on ZP resistance to proteolysis of nine different mammals

Effect on rabbit ZP

ZP of rabbit ovarian oocytes without exposure to OF were dissolved by pronase in < 3 min (0.04 ± 0.01 h) (Fig. 2). The ZP digestion time increased when the oocytes were treated with the OFs from five different species, particularly when sheep, goat and rabbit OF were used (Fig. 2).

Effect on mouse, rat and hamster ZP

The ZP of rodent oocytes not previously exposed to OF showed inter-specific differences in resistance to digestion by the enzyme pronase. The ZP digestion times of the different animals were as follows: mouse 0.04 ± 0.01 h (≈3 min); rat 0.06 ± 0.01 h (≈4 min) and hamster 24.4 ± 4.8 h (≈1 day). After treatments, the ZP of mouse and rat significantly increased their resistance to pronase digestion when incubated in the rabbit OF: 96.0 ± 8.0 h, ≈4 days in mouse (Fig. 3) and 207.4 ± 24.5 h in rat (≈8–9 days) (Fig. 4). Mouse ZP also

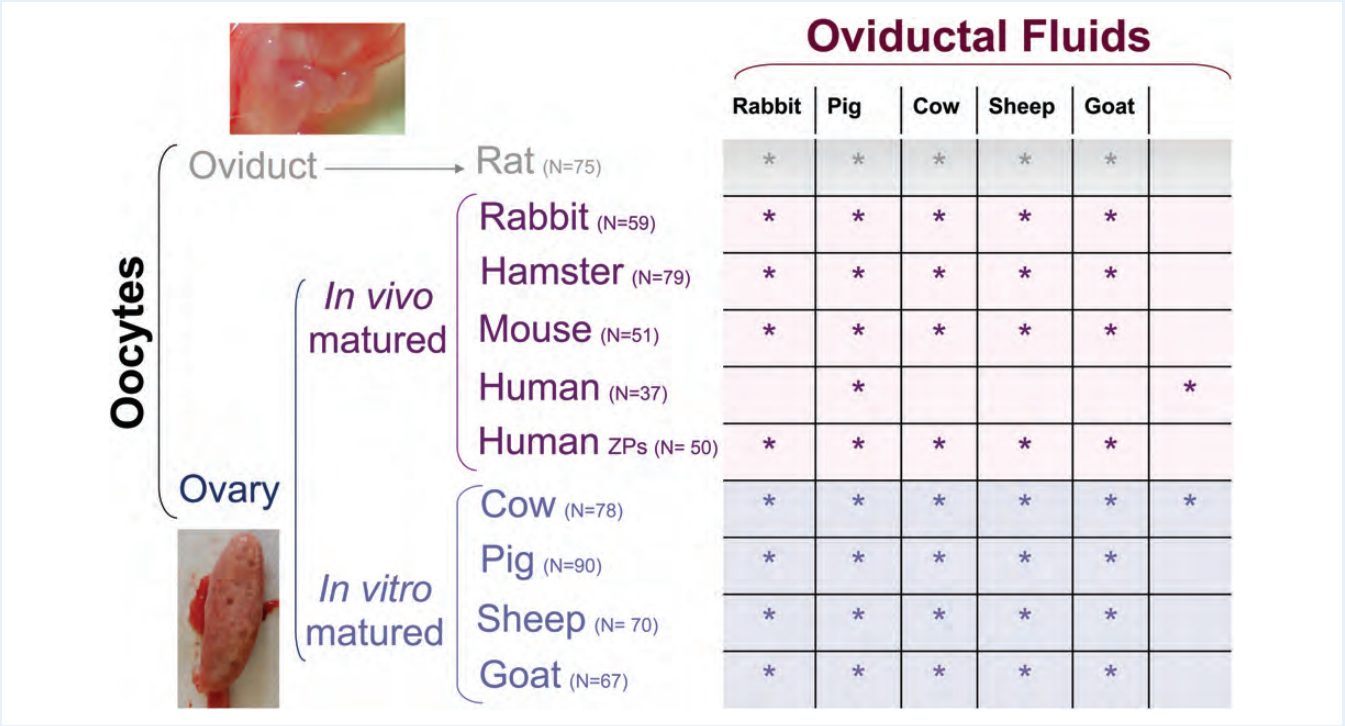

Figure 1 Experimental design. Oocytes of ovarian or oviductal origin and isolated ZPs (humans) were collected to assess ZP digestion time without and after incubation in different OFs. At least 10 oocytes per group were tested in three replicates.

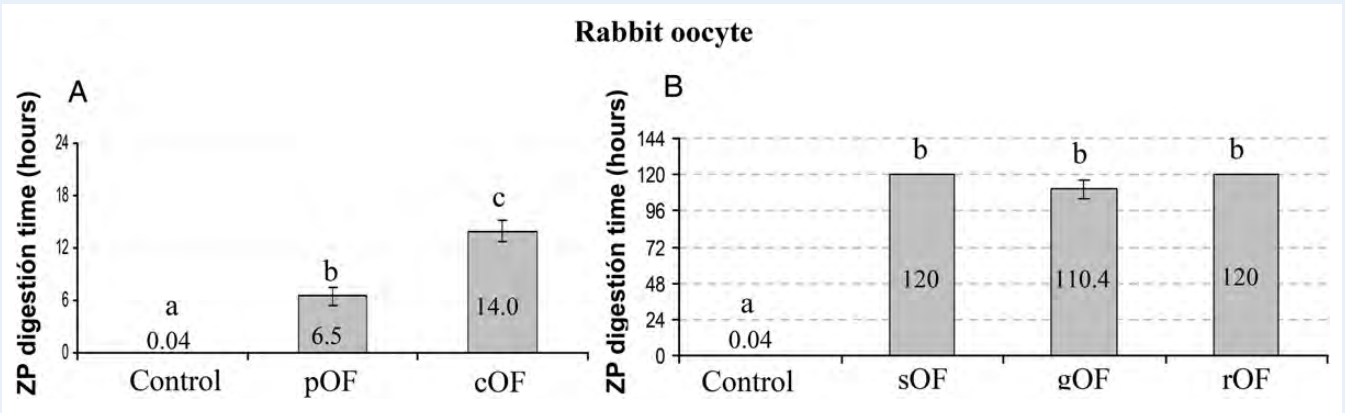

Figure 2 Effect of pOF and cOF (**A**) and of sOF, gOF and rOF (**B**), collected at the late follicular phase of the estrous cycle, on rabbit ZP resistance to protease digestion. Each bar represents the digestion time in hours (mean ± SEM) of oocytes incubated in the fluid for 30 min and later transferred to a pronase solution (0.5% wt/vol in DPBS). Different superscripts indicate significant differences among groups ($P < 0.05$).

became more resistant to proteolysis when the oocytes were treated with goat OF, taking 14.9 ± 1.6 h to be digested, while a smaller increase was noted after cow OF treatment (Fig. 3). However, rat ZP did not change after treatments with any secies except rabbit.

The ZP of hamster oocytes became more resistant to proteolytic digestion following incubation in pOF, sOF and rOF: 63.3 ± 13.0 h (≈2–3 days) in pig, 240.0 ± 3.6 h (≈10 days) in sheep and 683.3 ± 36.7 h (≈12 days) in rabbit OF (Fig. 5). It is important to mention that the dissolution pattern of hamster ZP was different from the other species because, while the outer part of the ZP was digested relatively quickly (within minutes), the inner part remained undigested for longer periods of time and this longer period was the value registered for hamster ZP hardening in the present study (Fig. 6).

Effect on human isolated ZPs and ZPs containing an oocyte

Isolated human ZPs not exposed to OF resisted enzymatic digestion for almost 2 min (0.03 ± 0.01 h; 1.8 ± 0.9 min). Treatment of these ZPs with OFs did not increase ZP digestion time except in the case of rabbit OF, where the increase was ~1 min (0.04 ± 0.01 h; 2.8 ± 0.1 min) (Fig. 7).

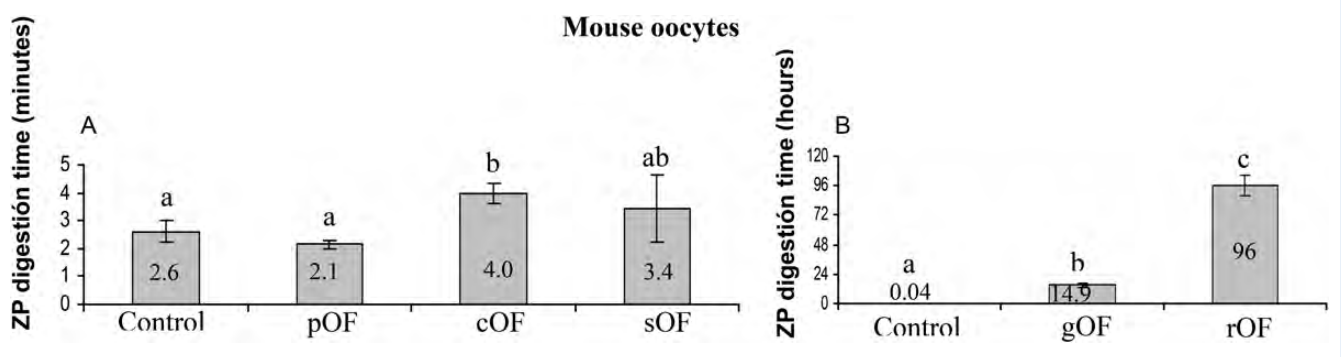

Figure 3 Effect of pOF, cOF and sOF (**A**) and of gOF and rOF (**B**), collected at the late follicular phase of the estrous cycle, on mouse ZP resistance to protease digestion. Each bar represents the digestion time (**A**) in minutes (mean ± SEM) or (**B**) in hours (mean ± SEM) of oocytes incubated in the fluid for 30 min and later transferred to a pronase solution (0.5% wt/vol in DPBS). Different superscripts indicate significant differences among groups ($P < 0.05$).

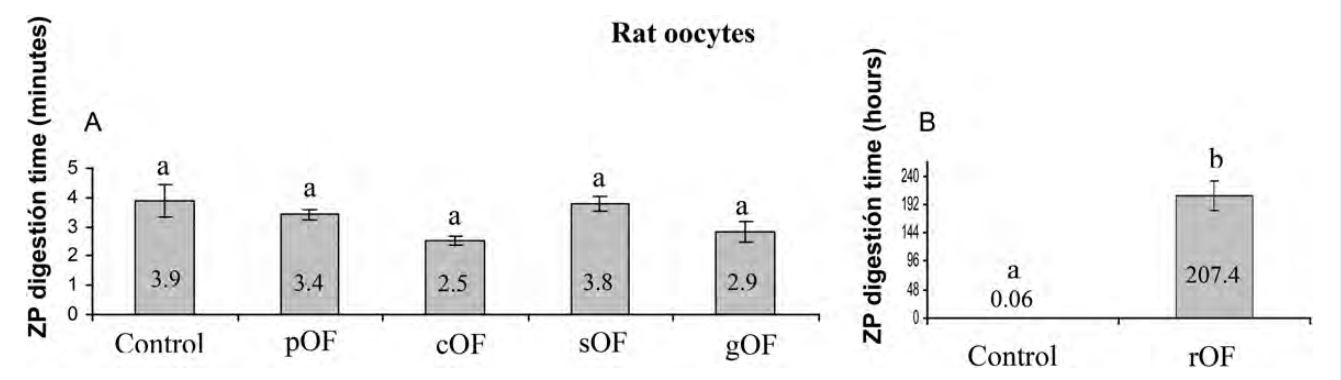

Figure 4 Effect of pOF, cOF, sOF and gOF (**A**) and of rabbit (rOF) (B), collected at the late follicular phase of the estrous cycle, on rat ZP resistance to protease digestion. Each bar represents the digestion (**A**) in minutes (mean ± SEM) or (**B**) in hours (mean ± SEM) of oocytes incubated in the fluid for 30 min and later transferred to pronase solution (0.5% wt/vol in DPBS). Different superscripts indicate significant differences among groups ($P < 0.05$).

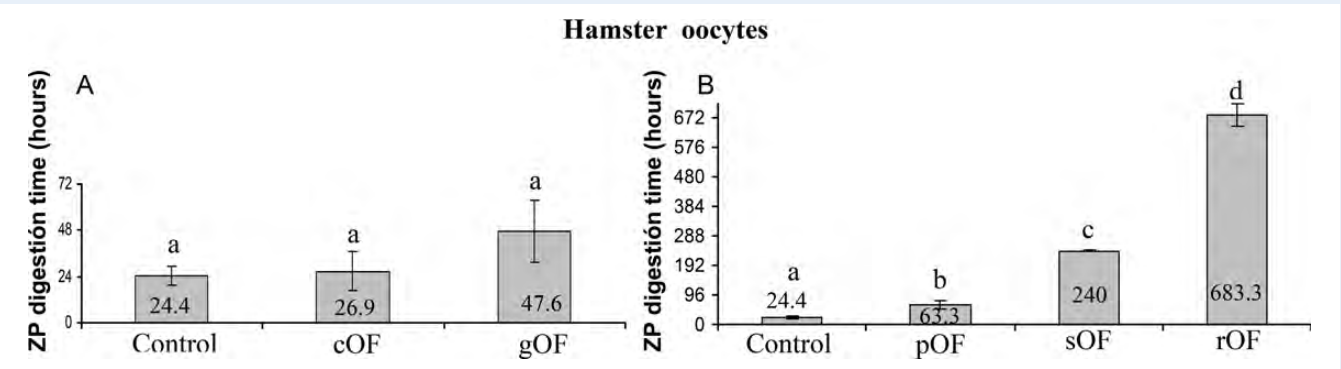

Figure 5 Effect of cOF and gOF (**A**) and pOF, sOF and rOF (**B**), collected at the late follicular phase of the estrous cycle, on hamster ZP resistance to protease digestion. Each bar represents the digestion time in hours (mean ± SEM) of oocytes incubated in the fluid for 30 min and later transferred to a pronase solution (0.5% wt/vol in DPBS). Different superscripts indicate significant differences among groups ($P < 0.05$).

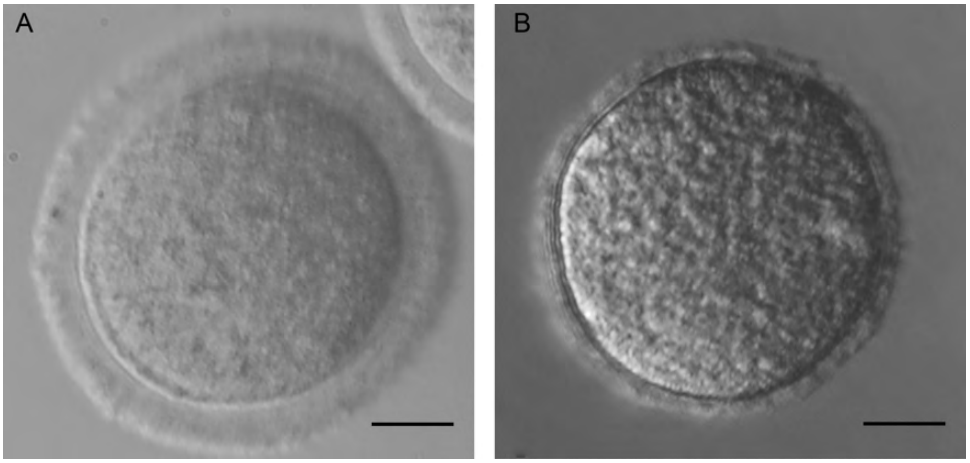

Figure 6 Hamster ovarian oocytes in two different situations. (**A**) Ovarian oocyte immediately before pronase treatment. (**B**) Ovarian oocyte incubated for 2 h in pronase solution. The outer region was digested, while the inner portion of the ZP remained visible. Scale bar = 20 μm.

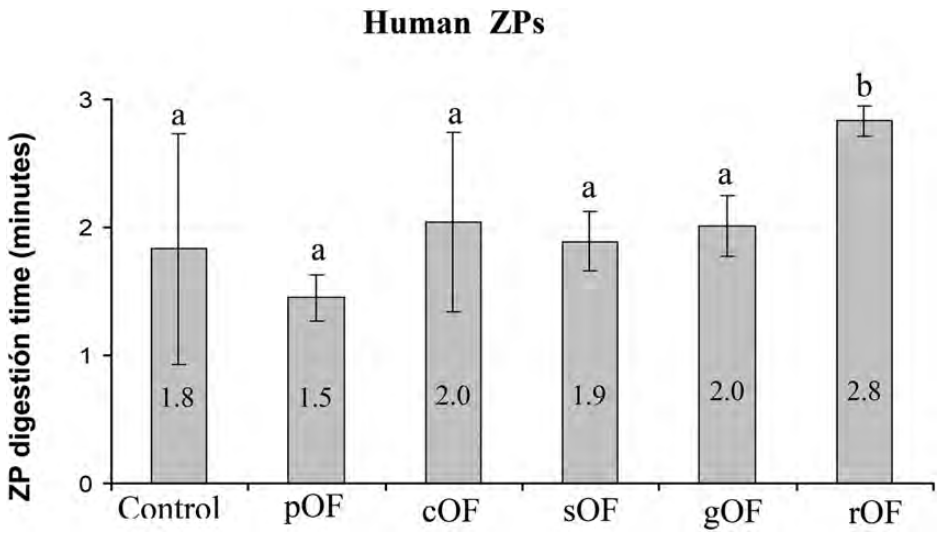

Figure 7 Effect of pOF, cOF, sOF, gOF and rOF, collected at the late follicular phase of the estrous cycle, on human ZP resistance to protease digestion. Each bar represents the digestion time in minutes (mean ± SEM) of oocytes incubated in the fluid for 30 min and later transferred to a pronase solution (0.5% wt/vol in DPBS). Different superscripts indicate significant differences among groups ($P < 0.05$).

To determine whether the lack of effect was caused by any alteration in ZP properties during the removal of the oocyte, full human oocytes enclosed in their respective ZPs were analyzed with OF from cow with its proven effect upon porcine oocytes (see later Fig. 9). In this case, again, cow OF failed to produce a significant increase in human ZP digestion time (Fig. 8).

Effect of human OF on porcine and human ZP resistance to proteolysis

Owing to the difficulty in obtaining human samples of OF in the rich estradiol phase, this experiment with only one sample from human oviduct was performed simply to confirm the results obtained with human oocytes incubated in the OFs from different animal species. Although statistical significance could not be assessed due to the limitation of the sample size, this experiment provided additional evidence concerning the lack of ZP hardening in humans. Thus, ZP from porcine and human oocytes took <3 min to be digested whether or not incubated for 30 min in human OF (Figs 8 and 9).

Effect on pig, cow, sheep and goat ZP

The ZP of porcine ovarian oocytes without exposure to OF showed low resistance (<4 min) to proteolytic digestion (0.05 ± 0.01 h), while treatment with the OFs increased ZP digestion time in all cases with the exception of human OF. This effect was more

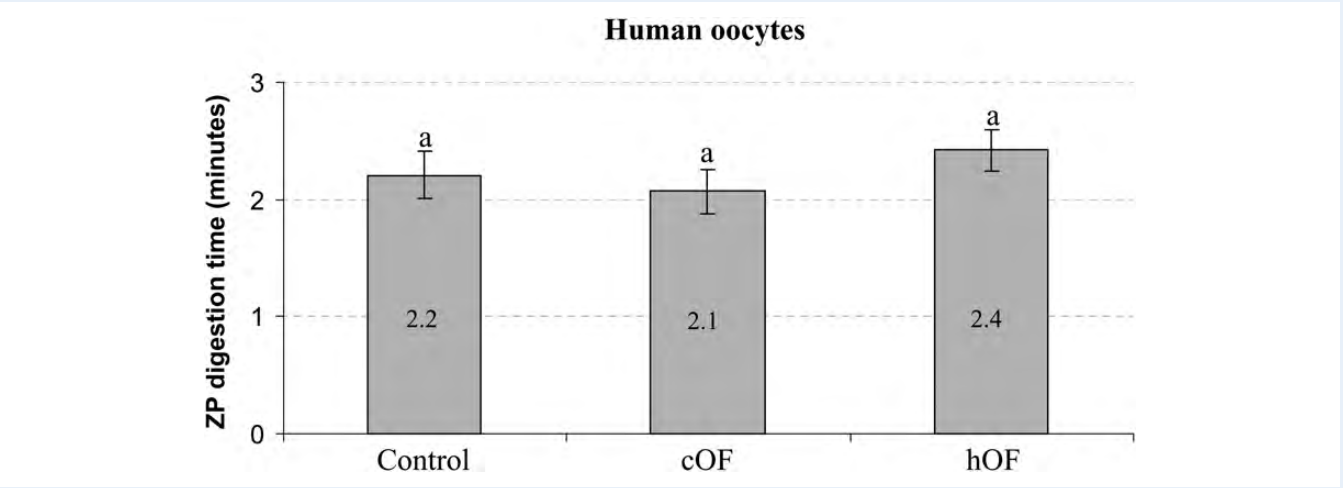

Figure 8 Effect of cOF, collected at the late follicular phase of the estrous cycle, and hOF on the human ZP resistance to protease digestion. Each bar represents the digestion time in minutes (mean ± SEM) of oocyte-surrounding ZP incubated in the fluid for 30 min and later transferred to a pronase solution (0.5% wt/vol in DPBS). Different superscripts indicate significant differences among groups ($P < 0.05$).

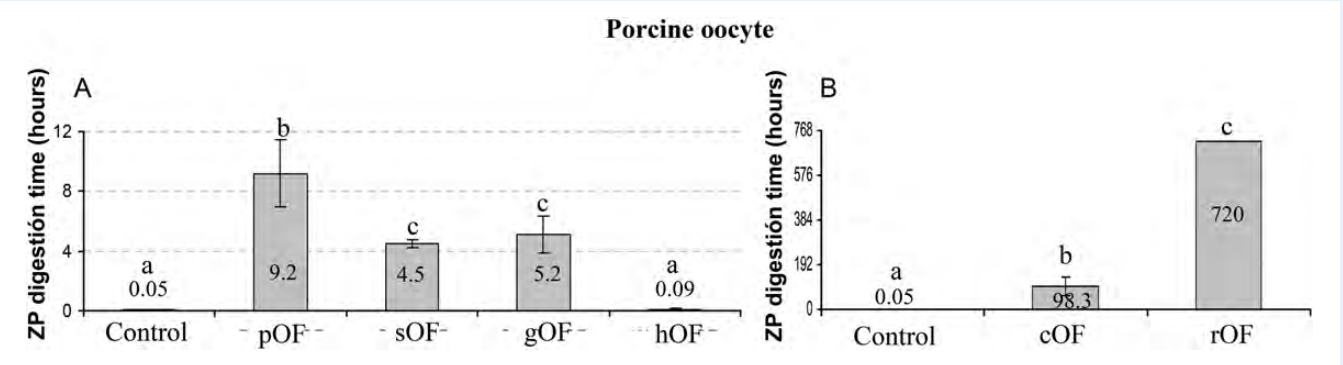

Figure 9 Effect of pOF, sOF, gOF and hOF (**A**) and of cOF and rOF (**B**), collected at the late follicular phase of the estrous cycle and secretory phase of the menstrual cycle (hOF), on pig ZP resistance to protease digestion. Each bar represents the digestion time in hours (mean ± SEM) of oocytes incubated in the fluid for 30 min and later transferred to a pronase solution (0.5% wt/vol in DPBS). Different superscripts indicate significant differences among groups ($P < 0.05$).

pronounced in the case of cow and rabbit OF (Fig. 9). The ZP of bovine ovarian oocytes showed a resistance to proteolytic digestion of <4 min (0.05 ± 0.01 h). In all cases, the ZP digestion time increased following treatment of the oocytes with the OFs. This effect was greater when the OFs from sheep, goat, cow and rabbit were used (Fig. 10). The resistance to proteolytic digestion of the ZP of ovine ovarian oocytes was <6 min (0.09 ± 0.01 h), and in all cases, the ZP digestion time was increased by treatment with the OFs. This effect was higher with cOF and rOF (Fig. 11). The resistance to proteolytic digestion of ZP of caprine ovarian oocytes was <4 min (0.06 ± 0.01 h) (Fig. 12). Treatment of these oocytes with the OFs increased ZP digestion time in different ways. No statistical differences were found when the oocytes were incubated with pOF and gOF, although the ZP digestion time changed from 0.7 h with pOF to 13.5 h with gOF. The effect was significant when considering the cOF, sOF and rOF (Fig. 12).

Discussion

This study shows that the ZPs of oocytes from different mammals increase their resistance to enzymatic digestion to a greater or lesser extent when they are incubated in the OF, with the exception of human ZP. In this last case, only the rOF caused a 1-min increase in ZP resistance to digestion. The biological significance of these findings is open to different interpretations, as discussed below.

Significance of enzymatic hardening

In this study, it is shown that the ZP of ovarian oocytes, whether matured *in vivo* or *in vitro*, without any contact with the oviduct, are sensitive to digestion by the enzyme pronase as revealed by the digestion periods of <6 min for eight of the nine species tested. The hamster was exceptional in this respect because the ZP took 24 h to be digested under the experimental conditions described. Previous

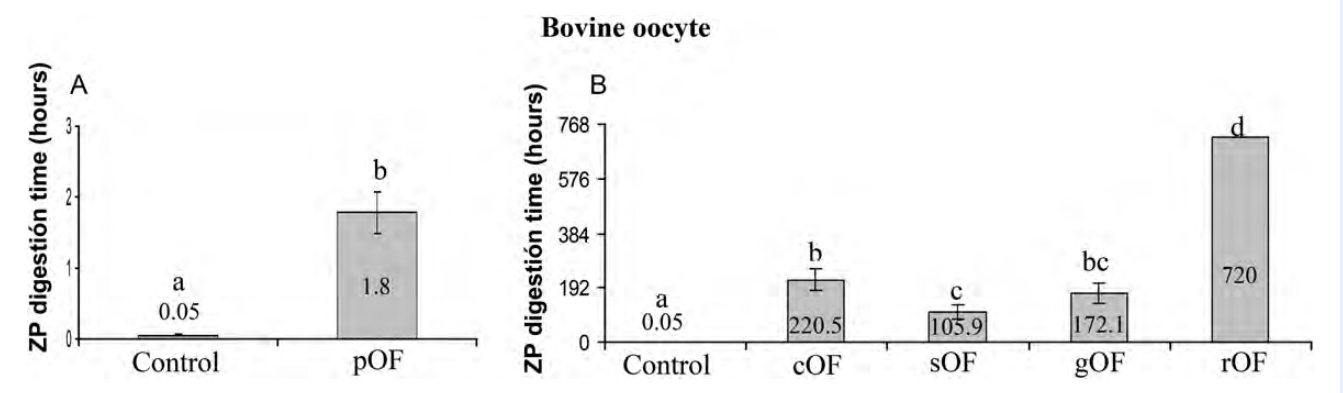

Figure 10 Effect of pOF (**A**) and of cOF, sOF, gOF and rOF (**B**), collected at the late follicular phase of the estrous cycle, on cow ZP resistance to protease digestion. Each bar represents the digestion time in hours (mean ± SEM) of oocytes incubated in the fluid for 30 min and later transferred to a pronase solution (0.5% wt/vol in DPBS). Different superscripts indicate significant differences among groups ($P < 0.05$).

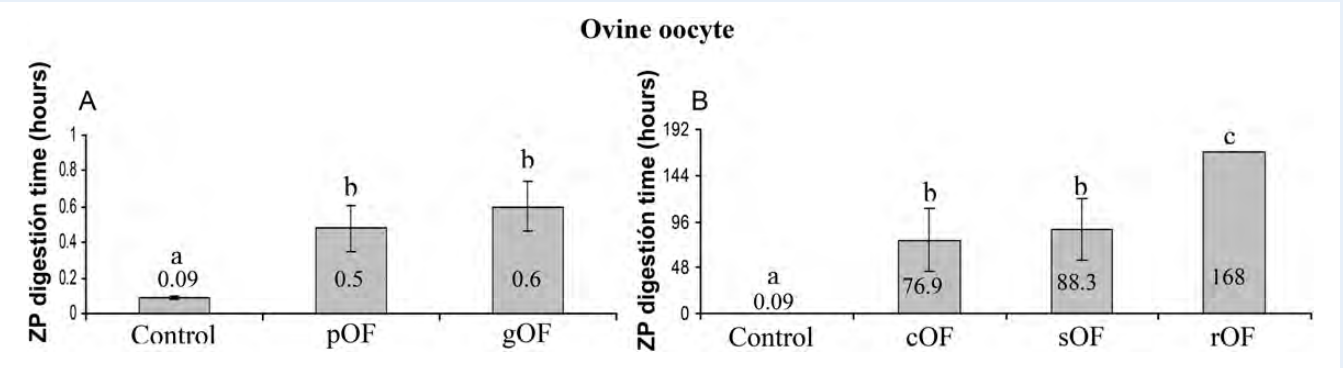

Figure 11 Effect of pOF and gOF (**A**) and of cOF, sOF and rOF (**B**), collected at the late follicular phase of the estrous cycle, on sheep ZP resistance to protease digestion. Each bar represents the digestion time in hours (mean ± SEM) of oocytes incubated in the fluid for 30 min and later transferred to a pronase solution (0.5% wt/vol in DPBS). Different superscripts indicate significant differences among groups ($P < 0.05$).

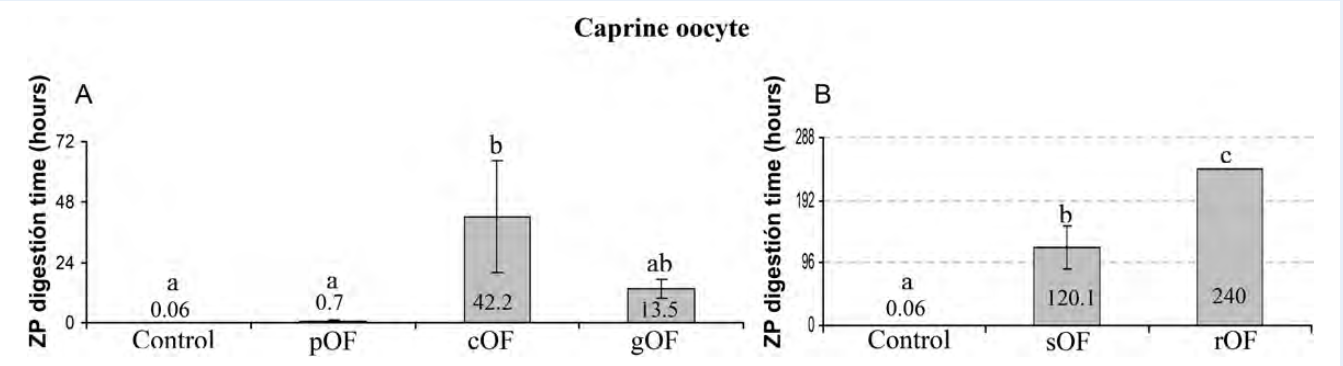

Figure 12 Effect of pOF, cOF and gOF (**A**) and od sOF and rOF (**B**), collected at the late follicular phase of the estrous cycle, on goat ZP resistance to protease digestion. Each bar represents the digestion time in hours (mean ± SEM) of oocytes incubated in the fluid for 30 min and later transferred to a pronase solution (0.5% wt/vol in DPBS). Different superscripts indicate significant differences among groups ($P < 0.05$).

studies in some of the species tested showed similar ZP digestion times for ovarian oocytes (mouse, rat, human, cow, sow, sheep and goat) (Trounson and Moore, 1974; Smorag and Katska, 1988; Katska *et al.*, 1989; Coy and Avilés 2010; Pradeep *et al.*, 2011).

With regard to hamster ZP, Yang and Yanagimachi (1989) reported a digestion time of ~6 min in 0.01% trypsin for ZPs from ovarian and oviductal oocytes. However, these data are not comparable with those in the present study because the experimental procedures

were different and because hamster oocytes showed a different pattern of ZP dissolution than the other species: the outer part of the ZP was digested relatively quickly, while the inner part remained stable for a long time. This is shown in Fig. 6 and could explain the differences between the present study and that of Yang and Yanagimachi's study (1989), in which only the digestion times of the outer part of the ZP seemed to have been assessed. Specific differences in the ZP structure or composition, or in the time necessary for the spermatozoa to contact this envelope after ovulation (Yanagimachi and Chang, 1961) could provide alternative explanations for the high resistance of hamster ZP to enzymatic digestion before being in contact with oviductal secretions, but further experiments are required to clarify this.

After treatment with homologous OF, ZP resistance to digestion increased in the case of the rabbit and all the ungulates except goat. Similarly, treatments with each of the heterologous fluids tested increased ZP digestion time for all the ungulate species and rabbit; the only exception to this was when goat oocytes were treated with porcine fluids. Human ZPs were not sensitive to OF, as discussed below.

Rabbit OF produced the largest increase in ZP resistance in all the species tested, indicating that the components of rabbit OF responsible for this effect are either more abundant or are more efficient than in the other species. The fact that rabbit ZP has been reported to remain equally penetrable despite being more resistant to proteases after fertilization (Overstreet and Bedford, 1974) could support the hypothesis that sperm penetration occurs more as a result of physical thrust than enzymatic digestion (Bedford, 2004). In general terms, the findings of the present study as a whole, which demonstrate the existence of oviductal induction of enzymatic ZP hardening in different ungulates and rodents, support this hypothesis and could be related to a sperm-selection mechanism in the oviduct that is dependent on the intrinsic ability of the spermatozoa to generate physical force.

In hamster which, as mentioned, is the species with the highest ZP digestion times in its ovarian oocytes (24 h), incubation in rOF increased the digestion time up to 683 h, while in human ZP, which did not show any hardening after treatment with any OF, the figure obtained was significantly different from that in the control oocytes, although this difference was only ~1 min. These two examples, at the opposite poles of the OF hardening effect, indicate that rOF is a good model to investigate the factors responsible for oviductal ZP hardening due to its strong and non-species-specific effect.

Regarding mouse and rat, although assays of their homologous fluid could not be performed due to the large number of animals required to obtain a suitable volume of OF, the experiments performed in the heterologous OFs provided interesting findings. Whereas mouse ZP became hardened after treatment with cOF, gOF and rOF, rat ZP was only sensitive to rOF. Since OVGP1, together with heparin-like glycosaminoglycans (GAGs), has been referred to as one of the OF components involved in enzymatic ZP hardening (Coy *et al.*, 2008; Pradeep *et al.*, 2011) and rat does not have OVGP1 (Tian *et al.*, 2009), the lack of effect of the OF from the different species tested on rat ZP was unexpected, assuming that heterologous OVGP1 from species other than rabbit could bind to rat ZP. In contrast, if it is assumed that the OVGP1 effect is species specific, the results obtained could indicate that other factors, besides OVGP1 and GAGs, participate in oviductal hardening and that these factors are more abundant or efficient in rabbit fluid. In addition, the existence of protein sequence and length differences between rabbit OVGP1 and those of other species could enable this protein to bind and modify rat ZP (Avilés *et al.*, 2010). A previous phylogenetic analysis showed that different regions are present in the OVGP1 of different mammals. OVGP1 lengths range from 475 in the rabbit (NP_001075574) to 721 amino acids in the mouse (Q62010) (Supplementary data, File S3). Taken together, these results suggest that oviductal maturation of oocytes as described by Coy *et al* (2008) is a process that (at least *in vitro*) is conserved in ungulates (at least in the pig, cow, sheep and goat), and that the factors involved in this mechanism are not specific to ungulate species, as they also affect rodents and rabbit.

The particular human situation

Human OF and human oocytes behaved differently from the other species studied. ZP enzymatic hardening, even when low numbers of oocytes were used, represents an evident 'all or nothing' mechanism, since the parameter considered (ZP digestion time) increases from a few minutes in untreated ovarian oocytes to several hours after treatment with OF. However, human oocytes did not change or, if they did, the scale of increase in ZP digestion time was only minutes. Neither in the case of isolated ZPs nor oocytes surrounding ZPs, did the digestion time change after incubation in OF. It could be argued that the human oocytes used in this study came from infertility clinics and cannot therefore be considered as 'healthy', but the results were consistent in the case of immature oocytes, those coming from fertilization failures and those that were fragmented. Finally, the sample of human OF, although unique, did not produce any detectable effects either on the pig or human oocytes, which is interesting bearing in mind that the porcine oocytes were the most sensitive to OF hardening among the species studied. Thus, it seems clear that the human is an exception with respect to this mechanism.

The explanation concerning the lack of effect of human OF, or the lack of sensitivity of the human ZP to hardening, cannot be attributed to an absence of OVGP1 because human OF contains OVGP1, and this OVGP1 binds to human (O'Day-Bowman *et al.*, 1996) and hamster (Reuter *et al.*, 1994) ZP. Consequently, the lack of effect may be due to differences in the OVGP1 glycoprotein sequence. In a previous study (Avilés *et al.*, 2010) it was observed that human OVGP1 and that from two other primates (chimpanzee and orangutan) have an additional region (region E) that was not present in the other species analyzed. The significance of this region of the OVGP1 is unknown and future studies are necessary to clarify this aspect. Two other aspects must to be taken into consideration: (i) the absence of some other, possibly relevant, factors present in the OF of other species and absent in human OF and (ii) differences in ZP composition. Human ZP is formed of four proteins (ZP1, ZP2, ZP3 and ZP4 (Lefievre *et al.*, 2004)) and these human ZP glycoproteins present a specific glycosylation pattern that has not been identified in other species (Jimenez-Movilla *et al.*, 2004; Pang *et al.*, 2011).

Physiologically, it is not strange that human OF did not produce ZP hardening. In the species where the effect has been most studied, such as pig and cow, it has been demonstrated that the hardening can be reversed when the recently fertilized oocyte is passing through the

oviduct into the uterus (Kolbe and Holtz, 2005; Coy *et al*., 2008). This 'softening' of the ZP in the preimplantational embryo has been related to its subsequent hatching but, in humans, the existence of this phenomenon in the oviduct probably means that the hardening remains stable in the uterus. This is so because, in contrast to the other mammalian species, the structural identity of the myometrium and myosalpinx suggests that the human uterus and utero-tubal junction (including the outer wall portion) can be considered as a unique morpho-functional entity (Muglia and Motta, 2001) and that mixing of uterine and oviduct fluids may be possible (Hunter, 1998, 2002). Such a combination of environments represents an advantage for ARTs, since it allows the transfer of gametes and early embryos into the human uterus. However, this same peculiarity was proposed as one cause of tubal ectopic implantation observed in primates but which does not occur in the other species analyzed (Hunter, 1998, 2002). In this sense, the present results might be considered as adding new information in the search for the reasons for tubal ectopic pregnancy in humans.

It is possible that the lack of ZP hardening by oviductal components in human oocytes may facilitate the adhesion of zygotes and preimplantational embryos to the oviductal epithelium, allowing their implantation. This hypothesis would be in agreement with the existence of the previously proposed factor preventing tubal implantation in rabbit (Moore *et al*., 1992) which, from the results of the present study, was the species with the highest ZP digestion times after contact with OF. It seems that the enzymatic hardening of the ZP is directly related to the deposition on this cover of different oviductal components that modify its properties. However, in humans, the lack of some of these components could increase the risk of adhesion between the ZP and the oviductal epithelial cells. Supporting this hypothesis, it has been proposed that selective production of protease inhibitors in the isthmus might act to prevent premature nidation of the embryo and that the increased resistance to proteolytic degradation of porcine oviductal ZP may represent a defence mechanism to avoid such degradation (Kouba *et al*., 2000). If human ZP lacks this mechanism, the risk of early implantation is, consequently, higher.

In conclusion, the present study shows that the pre-fertilization ZP hardening by oviductal components previously described in the pig and cow is common to other ungulate species and, while it could also affect rabbits and rodents, it does not exist in humans. The lack of this mechanism in humans may have further implications for the incidence of tubal ectopic pregnancies in this species.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgements

We thank William V. Holt and R.H.F. Hunter for their scientific advice and help with the English and Juan A. Carvajal, Darío Abril and Soledad Rodríguez for technical assistance with the collection of OF. We also thank Francisco Meseguer and his team in the Reina Sofia University Hospital in Murcia (Spain) for kindly providing human oviducts and Tahe fertility and IVI-Murcia clinic for providing human oocytes and ZP.

Authors' roles

I.M. collected the oocytes and performed the enzymatic analysis. I.M. and P.C. participated in the data analysis. M.A. and P.C. designed the study and conceived the project. All the authors discussed the results, commented on the manuscript and wrote the paper.

Funding

This work was supported by the Spanish Ministry of Science and Innovation and FEDER, Grant AGL2009-12512-C02-01-02.

Conflict of interest

None declared.

References

Austin CR. Observations on the penetration of the sperm in the mammalian egg. *Aust J Sci Res B* 1951;**4**:581–596.

Avilés M, Gutiérrez-Adán A, Coy P. Oviductal secretions: will they be key factors for the future ARTs? *Mol Hum Reprod* 2010;**16**:896–906.

Bedford JM. Enigmas of mammalian gamete form and function. *Biol Rev* 2004;**79**:429–460.

Boccaccio A, Frassanito MC, Lamberti L, Brunelli R, Maulucci G, Monaci M, Papi M, Pappalettere C, Parasassi T, Sylla L *et al.* Nanoscale characterization of the biomechanical hardening of bovine zona pellucida. *J R Soc Interface* 2012;**9**:2871–2882.

Burkitt M, Walker D, Romano DM, Fazeli A. Using computational modeling to investigate sperm navigation and behavior in the female reproductive tract. *Theriogenology* 2012;**77**:703–716.

Carrasco LC, Romar R, Avilés M, Gadea J, Coy P. Determination of glycosidase activity in porcine oviductal fluid at the different phases of the estrous cycle. *Reproduction* 2008a;**136**:833–842.

Carrasco LC, Coy P, Avilés M, Gadea J, Romar R. Glycosidase determination in bovine oviducal fluid at the follicular and luteal phases of the oestrous cycle. *Reproduction* 2008b;**20**:808–817.

Coy P, Gadea J, Romar R, Matás C, García E. Effect of in vitrofertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and in vitro development in pigs. *Reproduction* 2002; **124**:279–288.

Coy P, Cánovas S, Mondéjar I, Saavedra M, Romar R, Grullón L, Matás C, Avilés M. Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proc Natl Acad Sci USA* 2008; **105**:15809–15814.

Coy P, Avilés M. What controls polyspermy in mammals, the oviduct or the oocyte? *Biol Rev Camb Philos Soc* 2010;**85**:593–605.

Coy P, Lloyd R, Romar R, Satake N, Matás C, Gadea J, Holt W. Effects of porcine pre-ovulatory oviductal fluid on boar sperm function. *Theriogenology* 2010;**74**:632–642.

Funahashi H, Cantley T, Day B. Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. *Biol Reprod* 1997;**57**:49–53.

Gulyas BJ, Yuan LC. Cortical reaction and zona hardening in mouse oocytes following exposure to ethanol. *J Exp Zool* 1985;**233**:269–276.

Hunter RH. Have the Fallopian tubes a vital role in promoting fertility? *Acta Obstet Gynecol Scand* 1998;**77**:475–486.

Hunter RH. Tubal ectopic pregnancy: a patho-physiological explanation involving endometriosis. *Hum Reprod* 2002;**17**:1688–1691.

Jimenez-Movilla M, Avilés M, Gómez-Torres M, Fernández-Colom P, Castells M, de Juan J, Romeu A, Ballesta J. Carbohydrate analysis of the zona pellucida and cortical granules of human oocytes by means of ultrastructural cytochemistry. *Hum Reprod* 2004; **19**:1842–1855.

Katska L, Smorag Z, Gagda B. Dissolution of the zona pellucida of bovine oocytes after holding in homo and heterological oviduct. In: *Proceedings of 11th International Congress of Animal Reproduction and AI (ICARAI)*, Dublin, 1988, Vol. **3**, pp. 334–336.

Katska L, Kauffold P, Smorag Z, Duschinski U, Torner H, Kanitz W. Influence of hardening of the zona pellucida on in vitro fertilization of bovine oocytes. *Theriogenology* 1989;**32**:767–777.

Katska L, Kania G, Smorag Z, Wayda E, Plucienniczak G. Developmental capacity of bovine IVM/IVF oocytes with experimentally induced hardening of the zona pellucida. *Reprod Dom Anim* 1999;**34**:255–259.

Kolbe T, Holtz W. Differences in proteinase digestibility of the zona pellucida of in vivo and in vitro derived porcine oocytes and embryos. *Theriogenology* 2005;**63**:1695–1705.

Kouba AJ, Alvarez IM, Buhi WC. Identification and localization of plasminogen activator inhibitor-1 within the porcine oviduct. *Biol Reprod* 2000;**62**:501–510.

Lefievre L, Conner SJ, Salpekar A, Olufowobi O, Ashton P, Pavlovic B, Lenton W, Afnan M, Brewis IA, Monk M *et al*. Four zona pellucida glycoproteins are expressed in the human. *Hum Reprod* 2004; **19**:1580–1586.

Lloyd RE, Romar R, Matás C, Gutierrez-Adan A, Holt WV, Coy P. Effects of oviductal fluid on the development, quality and gene expression of porcine blastocyst produced in vitro. *Reproduction* 2009;**137**:679–687.

Moore GD, Eddy CA, Pauerstein CJ. Rabbit endosalpinx inhibits implantation in vitro. *Fertil Steril* 1992;**57**:902–907.

Muglia U, Motta PM. A new morpho-functional classification of the Fallopian tube based on its three-dimensional myoarchitecture. *Histol Histopathol* 2001;**16**:227–237.

O'Day-Bowman MB, Mavrogianis PA, Reuter LM, Johnson DE, Fazleabas AT, Verhage HG. Association of oviduct-specific glycoproteins with human and baboon (Papio anubis) ovarian oocytes and enhancement of human sperm binding to human hemizonae following in vitro incubation. *Biol Reprod* 1996;**54**:60–69.

Overstreet JW, Bedford JM. Comparison of the penetrability of the egg vestments in follicular oocytes, unfertilized and fertilized ova of the rabbit. *Dev Biol* 1974;**41**:185–192.

Pang PC, Chiu PC, Lee CL, Chang LY, Panico M, Morris HR, Haslam SM, Khoo KH, Clark GF, Yeung WS *et al*. Human sperm binding is mediated by the sialyl-Lewis(x) oligosaccharide on the zona pellucida. *Science* 2011;**333**:1761–1764.

Pradeep MA, Jagadeesh J, De AK, Kaushik JK, Malakar D, Kumar S, Dang AK, Das SK, Mohanty AK. Purification, sequence characterization and effect of goat oviduct-specific glycoprotein on in vitro embryo development. *Theriogenology* 2011;**75**:1005–1015.

Reuter LM, O'Day-Bowman MB, Mavrogianis PA, Fazleabas AT, Verhage HG. In vitro incubation of golden (Syrian) hamster ovarian oocytes and human sperm with a human oviduct specific glycoprotein. *Mol Reprod Dev* 1994;**38**:160–169.

Rizos D, Ramirez M, Pintado B, Lonergan P, Gutierrez-Adan A. Culture of bovine embryos in intermediate host oviducts with emphasis on the isolated mouse oviduct. *Theriogenology* 2010;**73**:777–785.

Smorag Z, Katska L. Reversible changes in dissolution of the zona pellucida of immature bovine oocytes. *Theriogenology* 1988;**30**:13–22.

Stetson I, Izquierdo-Rico MJ, Chevret P, Lorenzo PL, Ballesta J, Rebollar PG, Gutiérrez-Gallego R, Avilés M. Rabbit zona pellucida composition: a molecular, proteomic and phylogenetic approach. *J Proteomics* 2012;**75**:5920–5935.

Suarez SS. Regulation of sperm storage and movement in the mammalian oviduct. *Int J Dev Biol* 2008;**52**:455–462.

Tian X, Pascal G, Fouchecourt S, Pontarotti P, Monget P. Gene Birth, death, and divergence: the different scenarios of reproduction-related gene evolution. *Biol Reprod* 2009;**80**:616–621.

Trounson AO, Moore NW. The survival and development of sheep eggs following complete or partial removal of the zona pellucida. *J Reprod Fertil* 1974;**41**:97–105.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neil JD (eds). *The Physiology of Reproduction*. New York: Raven Press, 1994.

Yanagimachi R, Chang MC. Fertilizable life of golden hamster ova and their morphological changes at the time of losing fertilizability. *J Exp Zool* 1961;**148**:185–203.

Yang CH, Yanagimachi R. Differences between mature ovarian and oviductal oocytes: a study using the golden hamster. *Hum Reprod* 1989;**4**:63–71.