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Ubiquitin C-Terminal Hydrolase-Activity Is Involved in Sperm Acrosomal Function and Anti-Polyspermy Defense During Porcine Fertilization[1]

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ABSTRACT

The 26S proteasome, which is a multi-subunit protease with specificity for substrate proteins that are postranslationally modified by ubiquitination, has been implicated in acrosomal function and sperm-zona pellucida (ZP) penetration during mammalian fertilization. Ubiquitin C-terminal hydrolases (UCHs) are responsible for the removal of polyubiquitin chains during substrate priming for proteasomal proteolysis. The inhibition of deubiquitination increases the rate of proteasomal proteolysis. Consequently, we have hypothesized that inhibition of sperm acrosome-borne UCHs increases the rate of sperm-ZP penetration and polyspermy during porcine in vitro fertilization (IVF). Ubiquitin aldehyde (UA), which is a specific nonpermeating UCH inhibitor, significantly ($P < 0.05$) increased polyspermy during porcine IVF and reduced ($P < 0.05$) UCH enzymatic activity measured in motile boar spermatozoa using a specific fluorometric UCH substrate, ubiquitin-AMC. Antibodies against two closely related UCHs, UCHL1 and UCHL3, detected these UCHs in the oocyte cortex and on the sperm acrosome, respectively, and increased the rate of polyspermy during IVF, consistent with the UA-induced polyspermy surge. In the oocyte, UCHL3 was primarily associated with the meiotic spindle. Sperm-borne UCHL3 was localized to the acrosomal surface and coimmunoprecipitated with a peripheral acrosomal membrane protein, spermadhesin AQN1. Recombinant UCHs, UCHL3, and isopeptidase T reduced polyspermy when added to the fertilization medium. UCHL1 was detected in the oocyte cortex but not on the sperm surface, and was partially degraded 6–8 h after fertilization. Enucleated oocyte-somatic cell electrofusion caused polarized redistribution of cortical UCHL1. We conclude that sperm-acrosomal UCHs are involved in sperm-ZP interactions and antipolyspermy defense. Modulation of UCH activity could facilitate the management of polyspermy during IVF and provide insights into male infertility.

acrosome, deubiquitinating enzyme, fertilization, hydrolase, IVF, polyspermy, porcine, proteasome, sperm, spermadhesin, ubiquitin, ubiquitin C-terminal hydrolase

INTRODUCTION

Ubiquitin-proteasome-dependent proteolysis has been implicated in the control of mammalian gametogenesis and fertilization [1–4]. It plays an important role in selectively degrading and recycling proteins in many basic cellular processes, including but not limited to differentiation, cell cycle control, apoptosis, and immune response [5]. Ubiquitin is a highly conserved protein of 76 amino acid (AA) residues that can be covalently attached to cellular acceptor proteins that are primed for degradation by altered folding, oxidation, phosphorylation or glycosylation. For degradation by the 26S proteasome, ubiquitin binds covalently to its substrate proteins through a multi-step pathway, which requires the enzymatic activities of ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and the substrate-specific ubiquitin ligase E3. In order to target the substrate protein to the 26S proteasome, the binding of the first ubiquitin molecule to an internal lysine residue of the substrate protein is followed by tandem ligation of additional ubiquitin molecules, resulting in the formation of a polyubiquitin chain. After recognition by the 19S proteasomal regulatory complex, the polyubiquitin chain is removed and the substrate protein is translocated to the 20S proteasomal core, where it is degraded into small peptides.

Ubiquitin is released from the polyubiquitin chain or from a substrate protein through the action of deubiquitinating enzymes (DUBs) [6]. DUBs are cysteine proteases that specifically hydrolyze the amide bond immediately after the C-terminal residue (Gly76) of the ubiquitin molecule bound to an internal lysine of a substrate protein or to another ubiquitin within a polyubiquitin chain. The roles of DUBs are to maintain steady levels of unconjugated monoubiquitin and to stabilize ubiquitin-protein conjugates [7–9]. These activities facilitate the regeneration of monoubiquitin, recycling of ubiquitin, editing of polyubiquitin chains, and proteasome-dependent degradation. However, the inhibition of deubiquitination can actually increase the rate of proteasomal proteolysis [10]. When deubiquitinating activity is inhibited, the removal of multi-ubiquitin chains from the substrate fails and the whole

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complex of ubiquitinated protein is degraded by the 20S proteasomal core [10]. DUBs can be divided into ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific proteases (UBPs). UCHs remove short or flexible peptide chains from the carboxy-terminus of ubiquitin. The protein gene product 9.5 (PGP9.5 or UCHL1) belongs to a family of UCHs that regenerate monoubiquitin from ubiquitin-protein complexes or polyubiquitin chains by cleaving the amide linkage next to the C-terminal glycine of ubiquitin. UCHL1 has been identified in the rat brain and testis [11], and has been used as a marker of neurons [12] and spermatogonia [13]. UCHL1 shares 55% AA sequence identity with a closely related isozyme, UCHL3. UCHL1 and UCHL3 are highly expressed in mouse testes at the early and late stages of spermatogenesis, respectively [14, 15]. UCHL3, which is thought to be 200-times more enzymatically active than UCHL1 [16], is mainly expressed in spermatids [15].

Proteasomes are multicatalytic protease complexes that are found most frequently in the 26S and 20S configurations in various tissues and cells, thus representing the nonlysosomal proteolytic machinery of eukaryotic cells [17]. The sperm proteasome participates in the fertilization process from acrosomal exocytosis triggered by the egg coat glycoproteins to penetration of the vitelline coat and fusion with the egg plasma membrane [18]. The proteasome facilitates sperm binding and penetration through the vitelline coats of the eggs of a solitary ascidian (*Halocynthia roretzi*) [19–21]. The sperm proteasomes in *H. roretzi* are localized on the plasma membrane surface of the sperm head [22]. In the sea urchin *Strongylocentrouts intermedius,* the sperm proteasome is involved in Ca^{2+} channel activation leading to egg coat-induced acrosomal exocytosis [23]. Human sperm proteasomes are localized on the surface of the acrosomal plasma membrane and are involved in zona pellucida (ZP)/progesterone-induced acrosomal exocytosis and in the sustained phase of the Ca^{2+} influx provoked by progesterone [24, 25]. Sutovsky et al. [26] have inhibited sperm penetration through porcine ZP using reversible proteasomal inhibitors and antibodies specific for the 20S proteasomal core subunits. They detected proteasomes within the acrosomal matrix of intact boar spermatozoa and on the inner acrosomal membrane after ZP-induced acrosomal exocytosis. Thus, evidence has been accumulating that the 26S proteasome is a protease that is involved in acrosomal functions and digestion of the egg coat during both mammalian and nonmammalian fertilization [2, 27].

Fertilization in mammals is indicated by the formation of one male and one female pronucleus after the penetration of one spermatozoon into the oocyte. Such a constellation of paternal and maternal chromatin leads to normal embryonic development. However, penetration of more than one spermatozoon, which is called polyspermy, causes aberrant development and the death of the early embryo [28, 29]. The high incidence of polyspermy in porcine IVF systems remains to be explained. The polyspermy rate for in vivo porcine eggs is substantially higher (30–40%) than that for other species [29–31], and in the in vitro system, this rate often exceeds 50% [32–35]. The hardening of ZP associated with the exocytosis of cortical granules (cortical reaction) and cleavage of the ZP2 protein is thought to be a major component of the protective mechanism against polyspermy in the mouse [36]. However, Yanagimachi [37] has suggested that antipolyspermy control in the mouse occurs both at the zona level and at the oolema level, which explains why multiple spermatozoa can be seen in the perivitelline space of the murine zygote. Furthermore, Yanagimachi [37] has proposed that sperm-borne proteolytic activity is required for the initial digestion of zona surface, even if it may not be crucial for the subsequent penetration through the internal layers of the ZP. In ungulate and human eggs, the blockade of polyspermy occurs primarily at the level of the ZP [37–39]. Porcine polyspermy may be the result of a complete failure of cortical granule exocytosis in prematurely ovulated oocytes and delayed cortical granule exocytosis in oocytes that are matured in vitro [33]. Alternatively, oviductal glycoproteins and sperm acrosomal enzymes [40] may alter the oocyte ZP during natural fertilization, to prevent the binding and/or penetration of a large number of spermatozoa. The present study implicates the gametic UCHL1 and UCHL3 in the control of porcine polyspermy in vitro. While this study was under review, Sekiguchi et al. [41] reported high polyspermy rates in female gracile axonal dystrophy mutant mice (*Uchl1*^{gad}), which lack a functional UCHL1, and suggested that UCHL1 on the oocyte surface or in the oocyte cortex contributes to antipolyspermy defense in this species. The present study provides evidence that the sperm-acrosome-borne UCHs, including UCHL3, are involved in antipolyspermy defense during porcine fertilization. We also demonstrate that polyspermy can be alleviated by the addition of recombinant UCHL3 or related recombinant UCHs into the fertilization medium. Finally, we present the differential subcellular localizations of UCHL3 and UCHL1 in the porcine ovum and zygote, which are suggestive of the yet to be explored roles of these and other related UCHs in mammalian fertilization and preimplantation embryo development.

MATERIALS AND METHODS

Antibodies, Inhibitors, Substrates, and Recombinant Proteins

Mouse monoclonal antibody 31A3 directed against UCHL1/PGP9.5 (ab20559, Abcam, Cambridge, MA), which was raised against native PGP9.5 protein from the human brain, recognizes UCHL1 but not UCHL3. Rabbit anti-UCHL3 antiserum (LS-A8724; MBL, Woburn, MA) was raised and affinity-purified against a synthetic peptide that recognizes a unique C-terminal domain of human UCHL3. This antiserum recognizes UCHL3 but not UCHL1. Two different rabbit polyclonal sera against UCHL1/PGP9.5, AB1761 (Chemicon, Temecula, CA) and PG 9500 (Biomol, Plymouth Meeting, PA), were raised against purified human brain UCHL1/PGP9.5 and recognize both the UCHL1 and UCHL3 isoenzymes. Mouse monoclonal anti-ubiquitin antibody MK12–3 (MBL) was raised against purified bovine erythrocyte ubiquitin. Recombinant human UCHL3 [12] was purchased from Boston Biochem (Cambridge, MA). The catalytic activity of UCHL3 is estimated to be 200-times higher than that of UCHL1 [16]. Recombinant isopeptidase T (Ipase-T; Biomol) is a member of the ubiquitin C-terminal hydrolase family that hydrolyzes isopeptide bonds of polyubiquitin chains and regulates protein turnover through the ubiquitin-proteasome pathway [42]. Ubiquitin aldehyde (Biomol) is a potent and highly specific inhibitor of all ubiquitin C-terminal hydrolases, blocking the hydrolysis of polyubiquitin chains on substrate proteins and enhancing polyubiquitin chain accumulation [43]. Ubiquitin-AMC, which is composed of a full-length ubiquitin molecule linked at its C-terminus to the fluorescent dye 7-amido-methylcoumarin (AMC; Boston Biochem) [44], is a fluorogenic substrate with specificity for ubiquitin-C-terminal hydrolases. Agarose-immobilized, p62-derived UBA domain for affinity purification of ubiquitinated proteins [45] was purchased from Biomol. MG-132, which is a specific inhibitor of the chymotrypsin-like activity of the 20S proteasomal core [46] was purchased from Boston Biochem. As a negative control for fertilization experiments with the anti-UCH antibodies, several rabbit antisera that recognize major sperm head antigens not related to the ubiquitin-proteasome pathway were used at concentrations comparable with the concentrations of anti-UCH antibodies in the IVF media. These control sera recognize inner acrosomal membrane antigens IAM32 and IAM38 (the homologue of porcine ZPBP/SP38 protein; accession no. Q29108) [47, 48] and perinuclear theca antigen PT32/PAWP [49, 50] and have a content of sodium azide comparable to that of anti-UCH antibodies.

Unless mentioned otherwise, all other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

Collection, In Vitro Maturation, and Parthenogenetic Activation of Porcine Ova

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in a warm box (25–30°C). Ovaries were rinsed in 0.9% NaCl solution that contained 75 μg/ml penicillin G and 50 μg/ml streptomycin sulfate at 37.5°C. Cumulus oocyte complexes (COCs) were aspirated from antral follicles (3–6 mm in diameter) using an 18G needle attached to a 10-ml disposable syringe. COCs were washed three times in Hepes-buffered Tyrode lactate (TL-Hepes-PVA) medium that contained 0.1% (w/v) polyvinyl alcohol (PVA) and three times with the maturation medium [51]. A total of 50 COCs was transferred to 500 μl of the maturation medium that had been covered with mineral oil in a 4-well multidish (Nunc, Roskilde, Denmark) and equilibrated at 38.5°C in 5% CO_2 in air. The medium used for oocyte maturation was tissue culture medium 199 (TCM199; Gibco, Grand Island, NY) supplemented with 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg/ml LH, 0.5 μg/ml FSH, 10 ng/ml epidermal growth factor, 10% porcine follicular fluid, 75 μg/ml penicillin G, and 50 μg/ml streptomycin sulfate. After 22 h of culture, the oocytes were washed twice and cultured in TCM199 without LH and FSH for 22 h at 38.5°C in 5% CO_2 in air.

For the parthenogenesis experiments, cumulus-free, in vitro-matured oocytes were activated with 8% ethanol in TCM199 for 10 min, and then incubated with 4 mM 6-dimethylaminopurine (DMAP) in TCM199 for 6 h at 38.5°C in 5% CO_2 in air.

Semen Collection and Sperm Freezing

In each experiment, ejaculates from one Landrace boar were used under the guidance of approved Animal Care and Use (ACUC) protocols of the University of Missouri-Columbia (UM-C). The boars, which were bred and raised at the UM-C, were placed on a routine collection schedule of one collection per week. Sperm-rich portions of the ejaculates with greater than 85% sperm motility and normal acrosomes were used. The sperm-rich fraction was collected into a 250-ml insulated vacuum bottle. Semen was slowly cooled to room temperature (20°C) within 2 h after collection. Semen was transferred into 15-ml tubes, centrifuged at room temperature for 10 min at 800 × *g*, and the supernatant solution was removed. One volume of concentrated spermatozoa was resuspended in one volume of BF5 medium (the first dilution contained 5×10^8 sperm/ml) [52] at room temperature. Semen was cooled in a refrigerator to 5°C over a 2-h period and one volume of BF5 plus 2% glycerol diluent (the second diluent) was added to one volume of the cooled semen. Shallow wells for sperm freezing were made by placing warm metal beads on a block of dry ice. One hundred microliters of sperm suspension were loaded into each well and left for 1–2 min to solidify into a pellet. The pellets were placed in a liquid nitrogen tank for long-term storage.

In Vitro Fertilization and Culturing of Porcine Ova

After the completion of in vitro maturation, cumulus cells were removed with 0.1% hyaluronidase in TL-Hepes-PVA medium. Ova were washed three times with TL-Hepes-PVA medium and Tris-buffered (mTBM) medium [51] that contained 0.2% (w/v) BSA. Thereafter, 25–30 oocytes were placed into each of four 50-μl drops of the mTBM medium and covered with mineral oil in $35 \times 10\text{-mm}^2$ polystyrene culture dishes. The dishes were kept in the incubator for 30 min until spermatozoa were added for fertilization. A semen pellet was thawed in PBS that contained 0.1% PVA (PBS-PVA) at room temperature and centrifuged at 1500 × *g* through 60% and 40% isotonic Percoll layers for 10 min. The spermatozoa were resuspended and washed twice in PBS-PVA at 800 × *g* for 5 min. At the end of the washing procedure, the spermatozoa were resuspended in mTBM medium. After an appropriate dilution, 50 μl of this sperm suspension were added to 50 μl of the medium that contained oocytes at a final sperm concentration of 1×10^6 sperm/ml or 5×10^5 sperm/ml. In some experiments, antibodies against UCHL1/L3 or control antibodies (IAM32, 1:100; IAM38, 1:100; and PT32, 1:100) were added to fertilization drops at the time of sperm addition. Oocytes were coincubated with spermatozoa for 6 h at 38.5°C in 5% CO_2 in air. At 6 h after IVF, oocytes were transferred into 500 μl NCSU-23 culture medium that contained 0.4% BSA, for an additional culture period of 13–19 h.

Porcine Somatic Cell Nuclear Transfer

Mature oocytes were obtained from BoMed (Madison, WI) and cumulus-free (denuded) oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm in enucleation medium with a glass pipette of 30-μm diameter. The donor cells were injected into the perivitelline space of the oocyte through the same hole in the ZP, using the same pipette. Injected oocytes were placed between 0.2-mm-diameter platinum electrodes 1 mm apart in fusion medium (0.3 M mannitol, 1.0 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM Hepes), followed by culture in PZM-3 plus 0.3% BSA [53]. Fusion was induced with two DC pulses (1-sec interval) of 1.2 kV/cm for 30 μsec on a BTX Elector-Cell Manipulator 200 (BTX, San Diego, CA). The medium used for enucleation was TCM199 supplemented with 25 mM Hepes, 0.3% BSA, and 7.5 μg/mL cytochalasin B (CB), and the medium for injection was the same medium without CB [54, 55].

Mouse Gametes

Female ICR mice, bred in house at the University of Missouri-Columbia following approved ACUC protocols, were stimulated to superovulate by injecting 7.5 IU eCG, and injected 48 h later with 7.5 IU hCG. Oocytes were collected 14–18 h after hCG injection. The COCs from superovulated females were collected in mKR-Hepes medium. The cumulus cells were removed by repeatedly pipetting the COCs in 0.1 mg/ml hyaluronidase in mKR-Hepes. After washing thoroughly, the oocytes were transferred to acid Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.3 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2$, 0.3 mM $Na_2HPO_4 \cdot 7H_2O$ [pH 2.5], 0.1 mg/ml glucose, 4 mg/ml polyvinylpyrrolidone [PVP]) for a brief period. Oocytes were quickly transferred to a fresh drop of mKR-Hepes with 5 mg/ml PVP (M_r of 360 000) and pipetted repeatedly to remove the distended zona. Immature ova were isolated 48 h after eCG stimulation. Spermatozoa were isolated from the cauda epididymides of sexually mature ICR males by mincing the excised cauda epididymides in a Petri dish with mKR-Hepes medium and collection by centrifugation at 350 × *g*.

Immunofluorescence and Immunohistochemistry

Oocytes and embryos were fixed at the indicated time-points after IVM or IVF. The ZP was removed by a short incubation in TL-Hepes-PVA with 0.5% pronase. Oocytes were transferred into the first well of a nine-well Pyrex glass plate (Fisher Scientific, Brightwaters, NY) that was filled with 400 μl of warm (37°C) PBS, and 100 μl of 10% formaldehyde was slowly added to bring the final concentration of formaldehyde to 2% [56]. After 40 min of fixation at room temperature, the oocytes were washed in two wells of PBS, the fixative was removed, and the plates were wrapped in Handi-Wrap plastic wrap and stored for 1–7 days at 4°C. Prior to immunofluorescence processing, oocytes were permeabilized in PBS plus 0.1% Triton X-100 at room temperature for 40 min and blocked for 25 min in 0.1 M PBS that contained 5% normal goat serum and 0.1% Triton X-100. Primary antibody incubation was performed for 40 min with mouse antibodies and rabbit antisera (e.g., anti-UCHL1 and anti-UCHL3 antibodies), typically at 1:100 dilutions. After washing, the samples were incubated for 40 min with goat anti-rabbit IgG-TRITC (1:80 dilution; Zymed Inc., San Francisco, CA), alone or in combination with goat anti-mouse-FITC (for double-labeling experiments). The DNA stain DAPI (Molecular Probes, Eugene, OR) was added to the second antibody solution at a concentration of 2.5 μg/ml. Frozen-thawed spermatozoa were attached to poly-L-lysine-coated coverslips, and fixed, permeabilized, and immunolabeled as described for oocytes. Boar testicular tissues were obtained from fertile adult boars immediately after killing. Pieces of testicular tissue were minced under a dissecting microscope using fine forceps. Testicular cells were released into TL-Hepes medium, collected by centrifugation, and fixed on poly-L-lysine-coated coverslips, as described for spermatozoa [56]. Boar testicular tissue sections were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and further processed to obtain tissue sections by applying conventional histological techniques. Tissue sections were deparaffinized, rehydrated, quenched, and blocked as described previously [26], and processed with primary antibodies and fluorescently conjugated secondary antibodies as described above for oocytes. Multiple negative controls for both the immunocytochemical and immunohistochemical assays were performed by omitting the primary antibody and by incubation with preimmune rabbit serum in place of the primary antibody. In some experiments, the binding of anti-UCHL1/L3 antibody from the fertilization medium was visualized by the incubation of fixed, nonpermeabilized zygotes, which were fertilized in the presence of polyclonal anti-UCHL1/L3 antiserum in fertilization medium, with TRITC-conjugated goat anti-rabbit IgG (Zymed). Image acquisition was performed using a Nikon Eclipse 800 microscope (Nikon Instruments Inc., Melville, NY) with a Cool Snap camera (Roper Scientific, Tucson, AZ) and the MetaMorph software (Universal Imaging Corp., Downington, PA). Data were archived on CD-R compact disks, and edited using the Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA).

Electron Microscopy

Ova fertilized with or without rabbit anti-UCHL1/L3 antiserum (1:50 dilution; Biomol) were fixed in 2% formaldehyde. To compare the subcellular

localizations of sperm UCHs with that of the 26S proteasome, some ova were fertilized in the presence of a rabbit polyclonal antiserum against the 19S proteasomal regulatory complex subunit RPN12 (1:200 dilution; Biomol). Ova were blocked for 25 min in 0.1 M PBS that contained 5% normal goat serum, and incubated for 3 h with 12-nm colloidal gold-conjugated goat anti-rabbit IgG (1:5 dilution; Jackson Immunoresearch Laboratories Inc., West Grove, PA). Labeled ova were fixed in 0.6% glutaraldehyde plus 2% paraformaldehyde, postfixed in 1% osmium tetroxide, dehydrated, and embedded in PolyBed 812. Ultrathin sections were cut on a Leica Ultracut UCT ultramicrotome, stained with uranyl acetate and lead citrate, and photographed under a Jeol 1200 EX electron microscope. Photographs were scanned with a Umax Magic Scan flat-bed scanner (Umax Technologies Inc., Fremont, CA) and edited with Adobe Photoshop 7.0.

Western Blotting

For Western blotting, ZP-free matured oocytes (metaphase II) were used. Two sperm pellets were thawed, and approximately 1×10^9 sperm/ml were loaded per lane after extraction. In some experiments, mouse spermatozoa and testicular cell extracts were used as positive controls for the presence of UCHL1 and UCHL3. Porcine ova and spermatozoa were washed in warm PBS and boiled in loading buffer (50 mM Tris [pH 6.8], 150 mM NaCl, 2% SDS, 20% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue). Gel electrophoresis was performed in 4–20% gradient gels (PAGEr Gels; Cambrex Bio Science, Rockland, ME) by loading sperm extracts, or 50–100 oocyte extracts per lane, followed by transfer to PVDF membranes (Millipore Corp., Bedford, MA) using an Owl wet transfer system (Fisher Scientific, Houston, TX) at a constant 50V for 4 h. The membranes were sequentially incubated with 10% nonfat milk for 1 h and primary antibody (1:2000 dilution of anti-UCHL1/L3, anti-UCHL1 or anti-UCHL3) overnight. The polyclonal antisera were detected by incubation with HRP-conjugated goat anti-rabbit IgG (1:10 000 dilution) for 1 h, and the monoclonal anti-UCHL1 antibody was detected by HRP-conjugated goat anti-mouse IgG. The membranes were incubated with a chemiluminiscent substrate (SuperSignal; Pierce, Rockford, IL) and visualized by exposing to an x-ray film. Negative controls were performed using nonimmune sera.

Affinity Purification of Ubiquitinated UCH Species from Porcine Oocyte Extracts

Extracts of zona-free, in vitro-matured porcine ova were affinity purified by agarose-immobilized recombinant protein p62 (Biomol), which contains the UBA (ubiquitin-associated) domain that has high affinity for multi-ubiquitin chains (Biomol). The method of Sutovsky et al. [57] was used. Briefly, 300 ZP-free metaphase II ova were lysed as described for Western blotting, and incubated with p62-agarose for 20 min at room temperature. Complexes of p62 and ubiquitinated ooplasmic proteins were eluted in SDS-PAGE loading buffer, resolved on a 4–20% reducing gel, transferred onto a PVDF membrane, and probed with the polyclonal anti-UCHL1/L3 antisera, as described above.

Protein Immunoprecipitation and MALDI-TOF Mass Spectroscopy

Boar sperm extracts were immunoprecipitated with anti-UCHL1/L3 antibody using the Seize X Protein G immunoprecipitation Kit (Pierce) under nonreducing conditions, separated by SDS-PAGE, and stained with Coomassie blue. The immunoprecipitated band of ~13 kDa was excised carefully from the Coomassie blue-stained gel, destained, dehydrated, reduced with dithiothreitol (DTT), alkylated with iodoacetamide, washed, dehydrated, rehydrated with trypsin solution, and digested overnight with trypsin. The trypsin digest was dried, dissolved, and desalted on a Ziptip-C18 (Millipore). The peptides eluted from the Ziptip were analyzed by MALDI-TOF MS with alpha-cyano-4-hydroxycinnamic acid (CHCA) in the reflector mode over the mass range of 600–6000 Da. The MALDI-TOF MS spectra peak lists were obtained for the spectra after internal recalibration using trypsin autolysis fragment masses, computer baseline correction, noise removal, and peak deisotoping. The threshold for generating peak lists was set at 2% of the maximum observed peak area. The peak lists were submitted to Mascot for querying against the NCBInr mammalian database, and adjusted for trypsin digestion with no missed cleavage, fixed modification by carbamidomethylation, and variable modification by methionine oxidation. In the reversed immunoprecipitation experiment, boar sperm extracts were immunoprecipitated with an affinity-purified rabbit antiserum raised against boar spermadhesin AQN1 (Swiss Prot accession no. P26322) [58], and the precipitate was probed with epitope-specific anti-UCHL3 antibody (MBL). Appropriate positive and negative controls were included (sperm extract only, antibody-coated beads only, and antibody only).

UCH Activity Assay

In order to measure the enzymatic activity of sperm ubiquitin C-terminal hydrolases, ejaculated boar spermatozoa were sonicated to detach the sperm heads from the tails, and the heads were extracted [47, 59]. The sperm head extracts were assayed by spectrofluorometry (Synergy-HT multi-detection microplate reader; BioTek Instruments, Winooski, VT). Ubiquitin-AMC was used to monitor UCH activity, which releases the fluorogenic AMC component by specifically cleaving the bond between the C-terminus of ubiquitin and AMC. Aliquots (100-μl) of assay buffer (50 mM Hepes [pH 7.5], 0.5 mM EDTA , 0.1 mg/ml BSA, 1 mM DL-DTT) were added to individual wells of a 96-well plate (Costar; Corning) and supplemented with ubiquitin-AMC (final concentration, 1.25 μM) and sperm extracts (final concentration, 0.2 mg/ml). For the positive control, 25 nM of recombinant UCHL3 (Boston Biochem) replaced the sperm extracts. For the negative control, 15 μM PMSF, which is a general protease inhibitor that does not inhibit UCH activity, was added to sperm extracts and UCHL3. To inhibit the enzymatic activities of sperm UCHs, 0.2 mg/ml ubiquitin aldehyde was added to the wells with sperm extracts and UCHL3. An additional negative control was performed by incubating ubiquitin-AMC with trypsin, which would cleave ubiquitin internally without releasing the fluorogenic AMC dye from the C-terminus of ubiquitin. The aliquots were incubated for 30 min at 25°C, and subjected to excitation at 380 nm. Emission was recorded at 460 nm. The same experiments were repeated twice, and each measurement was carried out twice, respectively.

For the enzymatic assay of intact and sonicated spermatozoa, a sperm pellet obtained from a fresh ejaculate was thawed in assay buffer and centrifuged at $800 \times g$ for 5 min. After thawing, sperm motility was 30–50%. Spermatozoa were resuspended and washed in assay buffer at $800 \times g$ for 5 min. After washing, spermatozoa were diluted in assay buffer (final concentration of 5.0×10^6 sperm/ml), and divided into two equal-size aliquots, to be used as intact and sonicated spermatozoa. The diluted spermatozoa in one aliquot were sonicated using a Branson Sonifier (Branson Ultrasonics Co., Danbury, CT) at 20% amplitude for 1 min. The aliquots were incubated with ubiquitin-AMC and other assay components for 30 min at 39°C, and subjected to excitation at 380 nm. Emission was recorded at 460 nm. The same experiments were repeated twice, and each measurement was repeated three times, respectively.

Statistical Analysis

Analysis of variance (ANOVA) was performed using the general linear model (GLM) procedure of the Statistical Analysis System (SAS, Cary, NC). The Duncan multiple range test was used to compare values for individual treatments when the F-value was significant at $P < 0.05$.

RESULTS

Inhibition of Surface-Exposed Gametic UCHs Increases the Rates of Sperm-ZP Penetration and Polyspermy During Porcine IVF

The general theme of the ubiquitin-proteasome pathway is that the inhibition of substrate deubiquitination stimulates substrate proteolysis by the 20S proteasomal core [10]. In the presence of UCH inhibitors, the entire complex of substrate protein covalently linked to a multi-ubiquitin chain is degraded [10]. To further support our thesis that proteasomal proteolysis is involved in sperm-ZP interactions, we added a specific, nonpermeating UCH inhibitor, ubiquitin aldehyde (1–10 μM), to the IVF medium. The rate of monospermic fertilization was progressively reduced by increasing concentrations of UA. However, the polyspermy rate increased progressively with increasing UA concentration (Fig. 1A). This result indicates that UCH activity associated with sperm or the egg surface (UA is not cell-permeable) is necessary to regulate the rate of sperm penetration through the ZP.

Recombinant UCHs Reduce Polyspermy and Proportionally Increase the Rate of Monospermic Fertilization During IVF

If the inhibition of deubiquitination increases polyspermy, the addition of extrinsic UCHs should reverse this effect.

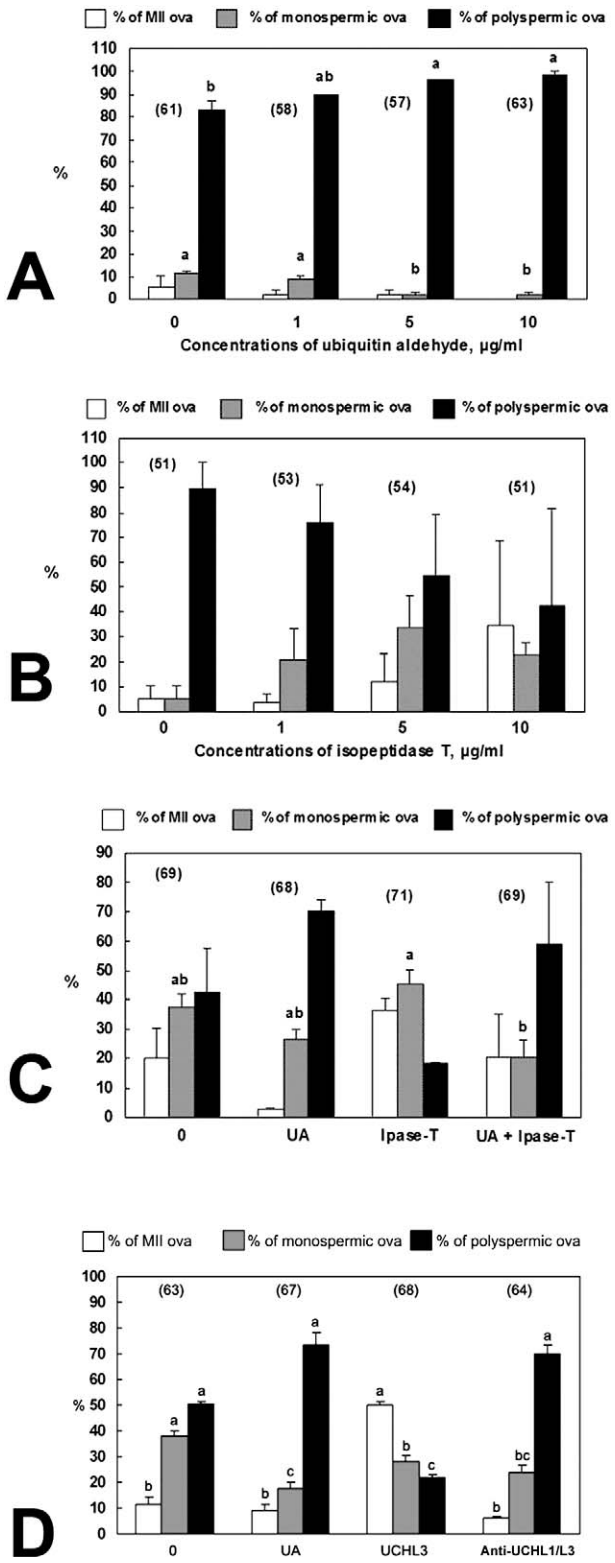

FIG. 1. Cell-impermeable UCH inhibitors increase polyspermy, while recombinant UCHs reduce polyspermy and maintain high rates of monospermic fertilization during porcine IVF. The unfertilized oocytes (metaphase II stage) are indicated by MII in the diagrams. Values are expressed as the mean percentages ± SEM. The numbers of inseminated ova are indicated in parentheses. Superscripts a, b, and c in each group of columns denote significant differences at $P < 0.05$. The average values from four replicates are shown. Effects of various concentrations of ubiquitin aldehyde (UA) (**A**), a specific inhibitor of ubiquitin C-terminal hydrolases, and recombinant isopeptidase T (Ipase-T) (**B**), an ubiquitin C-terminal hydrolase related to and synergistic with UCHL1 and UCHL3, on the fertilization parameters of porcine ova matured in vitro. **C**) Combined treatment with UA and Ipase-T. Both reagents were added at final concentrations of 5 µg/ml in the IVF media. The concentration of 0 µg/ml signifies a double-negative control. **D**) Comparison of monospermic and polyspermic fertilization rates in the presence of recombinant UCHL3 (2.5 nM), ubiquitin aldehyde (UA; 5 µg/ml), and polyclonal antisera that recognize UCHL1/L3 (1:50 dilution).

Contrary to the effect of UA, the percentage of polyspermy was indeed reduced significantly ($P < 0.05$) by the addition of recombinant Ipase-T (Fig. 1B). The UA and Ipase-T seemed to cancel each other out when added concomitantly to the fertilization medium (Fig. 1C). Even more pronounced was the reduction of polyspermy, which was paralleled by an increase in monospermic fertilization, observed during IVF in the presence of recombinant UCHL3 (Fig. 1D), which is thought to be among the most enzymatically active UCHs. Therefore, the addition of extrinsic UCHs to the IVF medium was applied to manage polyspermy during porcine IVF.

High Levels of UCH Activity Are Present on Boar Spermatozoa Surfaces

To measure UCH activity in boar spermatozoa, we took advantage of the specific fluorescent UCH substrate AMC. Ubiquitin-AMC fluoresces when cleaved into a free molecule of ubiquitin and a free, fluorescent molecule of AMC by UCHs, but not by trypsin or other proteases. Ubiquitin-AMC is not cell-permeable and therefore, it can only be used as a fluorescent substrate if the measured UCH enzymatic activity is present on the cell surface or if cell lysates are examined.

The assay was initially carried out using frozen-thawed boar sperm head extracts at 37°C in a 96-well plate reader. The high relative UCH enzymatic activity of the sperm extracts (33.5; no units) was comparable with that of recombinant UCHL3 (25.3) and not reduced significantly by PMSF (Fig. 2A). As expected, the UCH activities of the sperm extracts were reduced to a minimum both in the sperm extracts and UCHL3 assay wells by the specific UCH inhibitor, ubiquitin aldehyde (Fig. 2A). The same assay with identical positive and negative controls was carried out using freshly collected, motile boar spermatozoa, to show that UCH enzymatic activity resides on the sperm surface (Fig. 2B). As expected, sperm sonication prior to assaying, which partly removes acrosomes with acrosome-borne UCHs, reduced sperm UCH activity by more than 50% (Fig. 2B). We conclude that high levels of UCH enzymatic activity, comparable with the enzymatic activities of concentrated recombinant UCHs, are present in boar sperm extracts and on the surfaces of motile boar spermatozoa.

UCH Isozymes Are Present on the Boar Sperm Head Surface

We were able to measure the UCH activity in intact, live spermatozoa using ubiquitin-AMC (8.5 kDa), and to alter polyspermy during IVF using large molecules, such as ubiquitin aldehyde (8.5 kDa) and the recombinant UCHs (>20 kDa), none of which are cell-permeable. Therefore, we reasoned that the UCH activity involved in polyspermy control is most likely associated with the sperm or oocyte surface. Indeed, the addition of polyclonal anti-PGP9.5/UCHL1/L3 antibody (Biomol), which recognizes the closely related isozymes UCHL1 (also called PGP9.5; 223 AA) and UCHL3 (230 AA; 55% identity with AA sequence of UCHL1), increased the polyspermy rate in a dose-dependent fashion

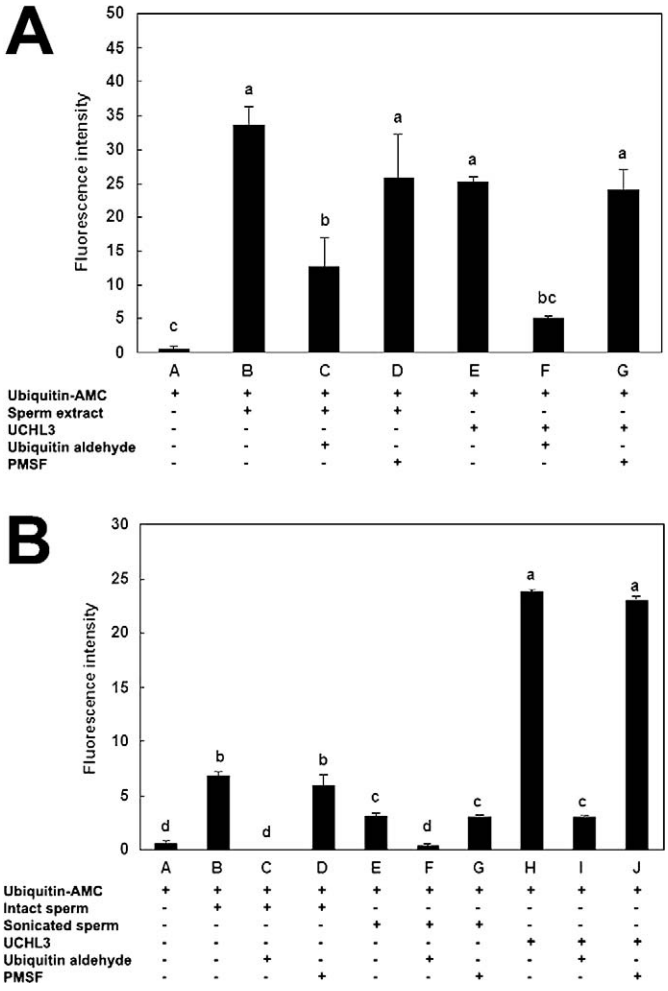

FIG. 2. High enzymatic activities of ubiquitin C-terminal hydrolases in boar sperm head extracts (**A**) and intact/sonicated spermatozoa (**B**), as measured using the specific fluorescent UCH substrate ubiquitin-AMC. The average values from four replicates are shown. Values are expressed as the mean ± SEM. Superscripts a, b, c, and d denote significant differences at $P < 0.05$. Recombinant UCHL3 was used as a positive control. Ubiquitin aldehyde (UA) was used as a negative control. PMSF, a general protease inhibitor with no effect on UCHs, was used as a secondary negative control.

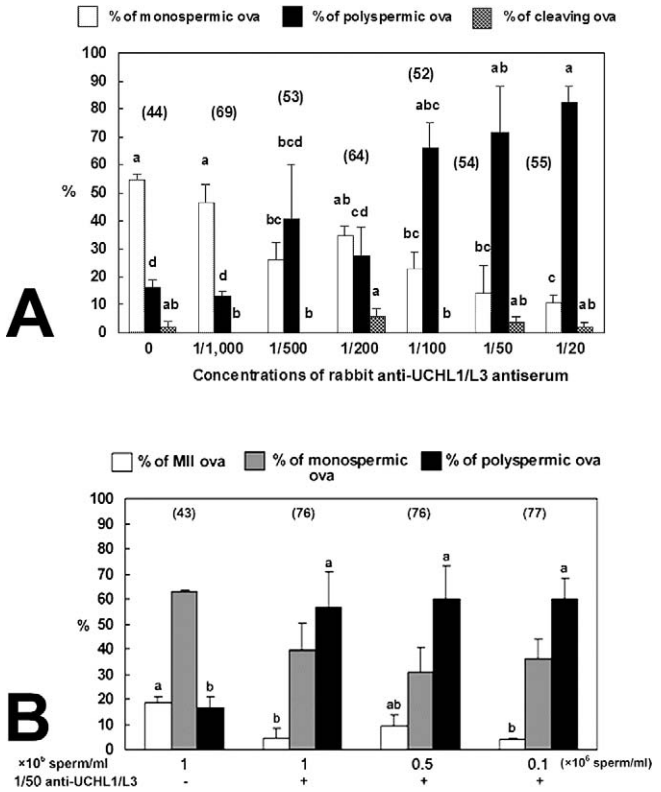

FIG. 3. UCH isozymes are exposed on the surface of the boar sperm head, as evidenced by the polyspermy surge induced by anti-UCH antibodies during IVF. **A**) Effects of progressively increasing concentrations of rabbit immune serum against UCHL1/L3 on the fertilization parameters of porcine ova matured in vitro. The experiments were repeated four times. Values are expressed as the mean percentage ± SEM. The numbers of inseminated ova are indicated in parentheses. Superscripts a, b, c, and d in each group of columns denote significant differences at $P < 0.05$. **B)** The action of anti-UCHL1/L3 antiserum (1:50 dilution in IVF medium) is not affected by sperm concentration.

(Fig. 3A; see also Fig. 1D). This polyspermy-enhancing effect was not affected by reduced sperm concentration at insemination (Fig. 3B), and it was not observed in control IVF experiments with nonimmune sera, unrelated immune sera or vehicle solutions that contained up to 1 mM sodium azide (Supplemental Fig. 1, A, B, and C, respectively, available online at www.biolreprod.org). Sperm motility was not affected and sperm-ZP binding was actually increased in the presence of the anti-UCHL1/L3 antibody (Supplemental Fig. 1D). The stimulatory effect of anti-UCHL1/L3 antibody on polyspermy was observed with both fresh (liquid) and frozen-thawed spermatozoa (Supplemental Fig. 1E). An unrelated polyclonal anti-UCHL1/L3 antiserum (Chemicon) had similar effects on fertilization and polyspermy rates (Supplemental Fig. 1F).

To determine where the immunoglobulins from the IVF medium attached to sperm or oocyte surfaces after incubation, we fixed the ova that were fertilized in the presence of rabbit anti-UCHL1/L3 antibody. We identified the immunoglobulin-binding sites by light microscopy after incubation with fluorescently conjugated anti-rabbit IgG (Fig. 4, A–E) and by electron microscopy after incubation of fixed ova with colloidal gold-conjugated anti-rabbit IgG (Fig. 5, A–I). Immunolabeling was detected exclusively on the surface of the acrosomal cap in the ZP-bound spermatozoa after IVF with the anti-UCHL1/L3-antibody but not after IVF with control sera and vehicle solutions (Fig. 4, C and E). Electron microscopy showed that the labeling was mainly associated with the outer surface of the acrosomal cap (Fig. 5, A, C, and D), with the vesicles produced by outer acrosomal membrane/plasma membrane vesiculation during acrosomal exocytosis (Fig. 5B), and with the same vesicles present in the rejected, ZP-bound acrosomal shrouds (Fig. 5, E and F). Comparable binding patterns were observed with polyclonal antiserum against the 19S proteasomal regulatory complex subunit RPN12 (Fig. 5G), but not with nonimmune rabbit sera (Fig. 5, H and I). The anti-UCHL1/L3 antisera were added to gametes during IVF and no permeabilization step was used in the subsequent processing. Thus, it is possible to conclude that the UCHs that are affected by specific UCH inhibitors and anti-UCH antibodies are primarily associated with the surfaces of acrosome-intact spermatozoa, and with the surfaces of acrosome-derived membrane vesicles and acrosomal ghosts in acrosome-reacted spermatozoa.

UCHL3 Is Present in the Sperm Acrosome and Interacts with Peripheral Acrosomal Plasma Membrane Proteins

Given that the anti-UCHL1/L3 antiserum recognizes two closely related (55% AA sequence identity) UCHs of identical

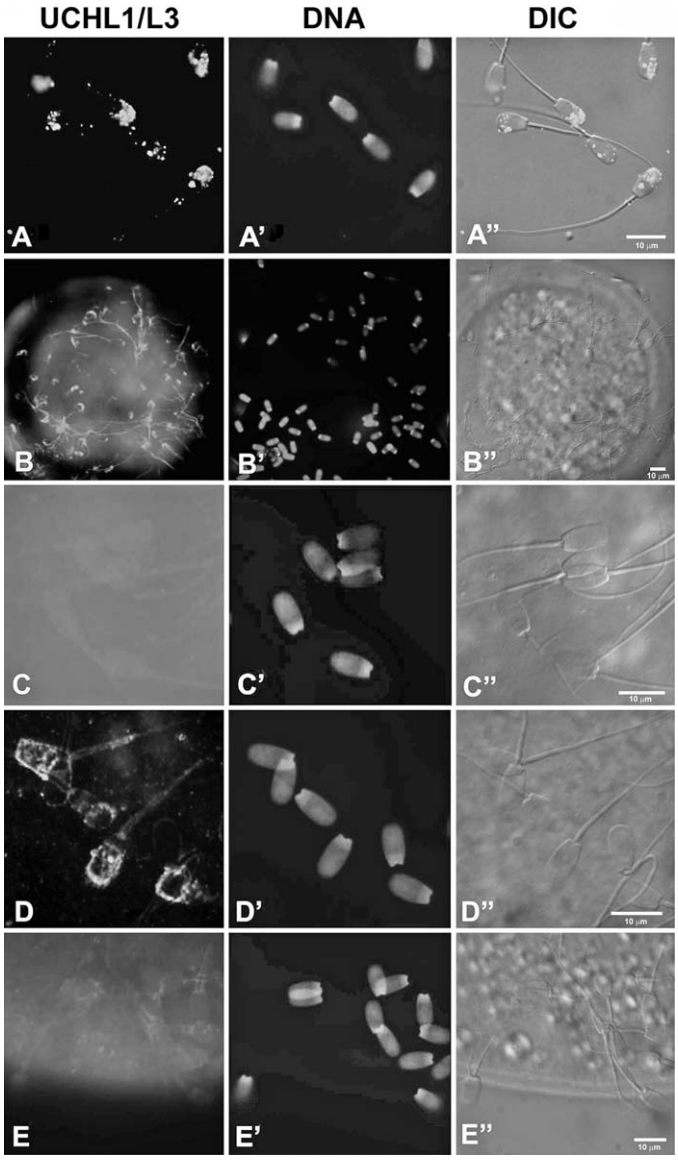

FIG. 4. Anti-UCH antibodies added to the IVF medium bind to the acrosomal surfaces of intact and acrosome-reacted spermatozoa bound to the ZP. Rabbit anti-UCHL1/L3 antiserum was added during fertilization and detected with fluorescent anti-rabbit IgG after the fixation of fertilized ova. **A–A″**) Control boar spermatozoa incubated with anti-UCHL1/L3 antiserum followed by fluorescent goat anti-rabbit IgG (GAR-TRITC). **B–B″**) Ova fertilized in the presence of anti-UCHL1/L3 antiserum and incubated with GAR-TRITC after fixation. **C–C″**) Ova fertilized in the presence of a nonimmune rabbit serum and processed with GAR-TRITC after fixation. **D–D″**) Detail of spermatozoa on the ZP surface of ova inseminated in the presence of the anti-UCHL1/L3 antiserum. **E–E″**) Control fertilized ova (no anti-UCHL1/L3 antiserum or nonimmune serum during IVF). The DNA in all panels is counterstained with DAPI.

size (~24 kDa), we examined whether the UCH activity in the acrosomal cap could be attributed to one of these UCHs (UCHL1 or UCHL3). In the mouse, the UCHL1 and UCHL3 proteins are abundant in the testis, allowing us to use mouse testicular cell and sperm extracts as positive controls for our anti-UCH antisera. UCHL1 is primarily found in mouse spermatogonia and UCHL3 is found in the spermatocytes and spermatids [15]. Using monospecific anti-UCHL1 and anti-UCHL3 antibodies, we detected both UCHL1 and UCHL3 (immunoreactive 24-kDa bands) in boar testicular cells, whereas only the UCHL3 immunoreactivity was detected in the ejaculated boar spermatozoa by Western blotting (Fig. 6, A and B) and immunocytochemistry (Fig. 6, C and D). Acrosomal localization of UCHL3 was performed both in spermatozoa permeabilized with Triton X-100 (Supplemental Fig. 2; also shows the negative control) and without permeabilization (Fig. 6D), to confirm that UCHL3 is exposed on the acrosomal surfaces of nonpermeabilized, ejaculated spermatozoa. The pattern of UCH distribution in the testis was confirmed by immunohistochemical localization of UCHL1 mainly to spermatogonia (Fig. 6E) and of UCHL3 to the round spermatids (acrosomal cap) and elongating spermatids (caudal manchette and acrosome) (Fig. 6, F, G, and H). UCHL3 was also detected prominently in the Sertoli cell cytoplasm and Sertoli cell sperm head junctional complexes (Fig. 6, I and J).

We further investigated whether UCHs present on the acrosomal surface interact with any known acrosomal surface proteins. We applied the polyclonal anti-UCHL1/L3 antiserum (Fig. 7A) to immunoprecipitate proteins that interact with sperm acrosomal UCHs. Immunoprecipitation under nonreducing conditions followed by MALDI-TOF protein identification showed that acrosomal UCHs in the ejaculated boar spermatozoa interacted with spermadhesin AQN1 (Fig. 7, B and C). AQN1 is a well-known peripheral membrane protein of the acrosomal plasma membrane that migrates on SDS-PAGE as a typical triplet of bands in the 14–16 kDa range [58, 60, 61]. Confirming the specificity of immunoprecipitation, back-precipitation with an affinity-purified anti-AQN1 antibody [58] resulted in the coimmunoprecipitation of UCHL3 (Fig. 7D). The anti-AQN1 antibody used for this reversed precipitation experiment localized AQN1 to the boar sperm acrosome, further confirming the specificity of the UCHL3-AQN1 interaction on the acrosomal surface (Fig. 7, E and F). Taken together, these data indicate that UCHL3 is present on the acrosomal surfaces of boar spermatozoa, and that it is capable of interacting with the ZP surface and with peripheral acrosomal membrane proteins. UCHL3 could be absorbed onto the outer acrosomal membrane during the haploid phase of spermatogenesis and could be sourced from Golgi-derived proacrosomal vesicles and from endocytotic vesicles that incorporate Sertoli cell-secreted proteins [62].

Oocyte-Secreted UCHs Are Not Necessary for Antipolyspermy Defense During Porcine IVF

Predating the recently published work on the polyspermic UCHL1-deficient *Uchl1*^{gad} mutant mouse [41], we examined whether UCHs could be secreted by the porcine ovum and participate in antipolyspermy defense. Sekiguchi et al. [42] have suggested that UCHL1 is located in the mouse oocyte cortex and on the surface of the mouse oolemma, and that it is necessary for antipolyspermy defense in the mouse. Using the specific anti-UCHL1 and anti-UCHL3 antibodies, we found that high levels of UCH immunoreactivity were present in both the porcine (Fig. 8, A–D) and murine (Fig. 8, E and F; see Supplemental Fig. 3 for the full dataset) oocyte cortices. This cortical labeling was not affected by fixation-induced blebbing of the oolemma, which is typical of porcine ova. We observed that a granule-like material that contained UCHs was secreted into the perivitelline space during mouse oocyte maturation (Fig. 8F), but not during porcine oocyte maturation (Fig. 8, A and B). Some immunoreactive material was observed in the perivitelline space of fertilized porcine ova (Supplemental Fig. 4, A and B), whereas when the fertilized ova were examined without permeabilization, there was no reactivity on the oocyte surface (Fig. 8D). UCHL3 was confined to the meiotic spindle of metaphase II ova (Fig. 8A) and distributed diffusely in the

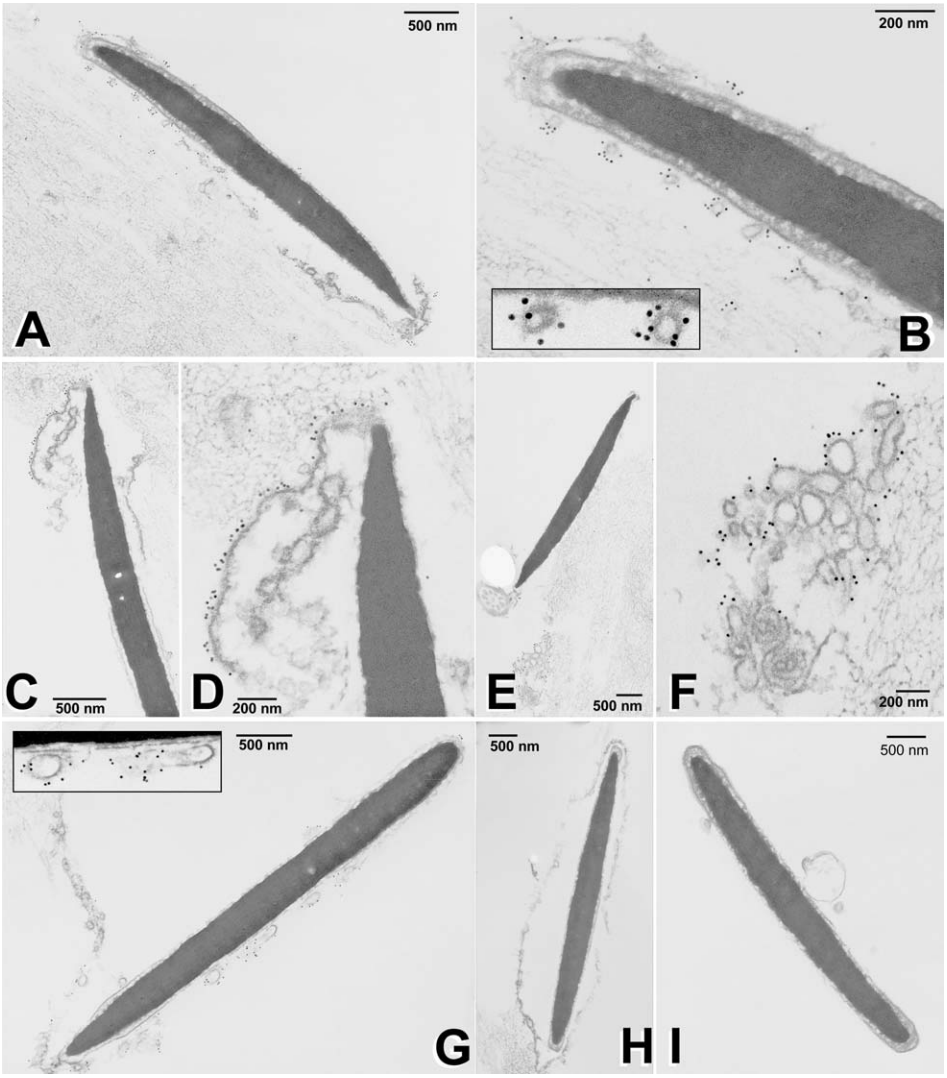

FIG. 5. Binding of anti-UCH antisera to fertilizing spermatozoa occurs primarily on the acrosomal surfaces of the acrosome-intact spermatozoa (**C**, **D**) and on the surfaces of acrosomal vesicles (**A**, **B**) formed by the fusion of acrosomal and plasma membranes after acrosomal exocytosis. Some of these vesicles remain associated with the sperm head surface (**A**, **B**), while most of the vesicles constitute the rejected acrosomal shroud (**E**, **F**). Antibody against the subunit RPN12 of the 19S proteasomal regulatory complex was used as a positive control (**G**) and showed localization consistent with that of acrosomal UCHs. Porcine ova were inseminated in the presence of anti-UCHL1/L3 antiserum (or anti-RPN12 antibody), fixed, and incubated with colloidal gold-conjugated goat anti-rabbit IgG. Negative controls fertilized in the presence of nonimmune sera are shown in panels **H** and **I**.

zygotic cytoplasm after fertilization (Fig. 8, B and C). Intriguingly, the fusion of a porcine ovum with a somatic cell (fetal fibroblast) caused the formation of a large UCHL1-free cortical domain, which probably corresponds to an oocyte cortex area that incorporates plasma membrane contributed by the donor cell (Fig. 8G). This polarized redistribution was not observed for UCHL3, which was evenly distributed throughout the ooplasm of reconstructed zygotes (Fig. 8G). Overall, the cortical localization of UCHL1 and spindle-localization of UCHL3 seem to be evolutionarily conserved, as has been also found for bovine ova (Supplemental Fig. 5).

Biochemical analysis with the polyclonal anti-UCHL1/L3 antibody demonstrated that the UCH isozymes present in the oocyte, most of which were most likely contributed by UCHL1 [63, 64], were turned over by the ubiquitin-proteasome pathway and their content was diminished after oocyte activation/fertilization (Fig. 8, H–I). Ubiquitination of ooplasmic UCHs was suggested by the appearance of additional immunoreactive bands above the expected 24-kDa UCHL1/L3 band in Western blots of metaphase II ova (Fig. 8H, lane 1). Ubiquitination increases the mass of substrate proteins by stable covalent ligation of multi-ubiquitin chains (multiples of the 8.5-kDa molecular mass of monoubiquitin) to the internal lysine residues of the substrate. These high-molecular-mass UCH bands were greatly diminished after oocyte activation induced by fertilization or parthenogenetic activation (Fig. 8H, lanes 2 and 3, respectively, and Fig. 8I), although some high-molecular-mass UCH bands accumulated in fertilized ova that were treated with the specific, cell-permeable proteasomal inhibitor MG-132 (Fig. 8H, lane 4). Ubiquitinated UCH bands were enriched in the ooplasmic fraction from which ubiquitinated proteins were affinity-purified by agarose-immobilized, recombinant ubiquitin-binding protein p62 (Fig. 8J, lane 1).

Finally, we examined whether the secretion of UCHs via cortical granule exocytosis during oocyte activation contributes to antipolyspermy defense during porcine IVF. We did not observe any changes in the fertilization rates when ova were fertilized after preincubation with anti-UCH antiserum prior to IVF (Supplemental Fig. 5C). Not even parthenogenetic activation of ova prior to IVF and antibody preincubation were sufficient to alter fertilization and polyspermy rates. In this experiment, we activated the ova parthenogenetically, then preincubated them with anti-UCH antibodies and fertilized them in the absence or presence of anti-UCH antibodies (Fig. 9A, additional repeats in Supplemental Fig. 4, C and D). None of these treatments had any substantial effect on polyspermy rates, and the differences in the numbers of oolemma-bound accessory spermatozoa per ovum were consistent with the action of ubiquitin aldehyde and anti-UCH antibodies on sperm acrosomal UCHs during IVF (Fig. 9B). We conclude that oocyte-secreted UCHs are not a primary tool for antipolyspermy defense in the pig. It remains possible that UCHL1 on

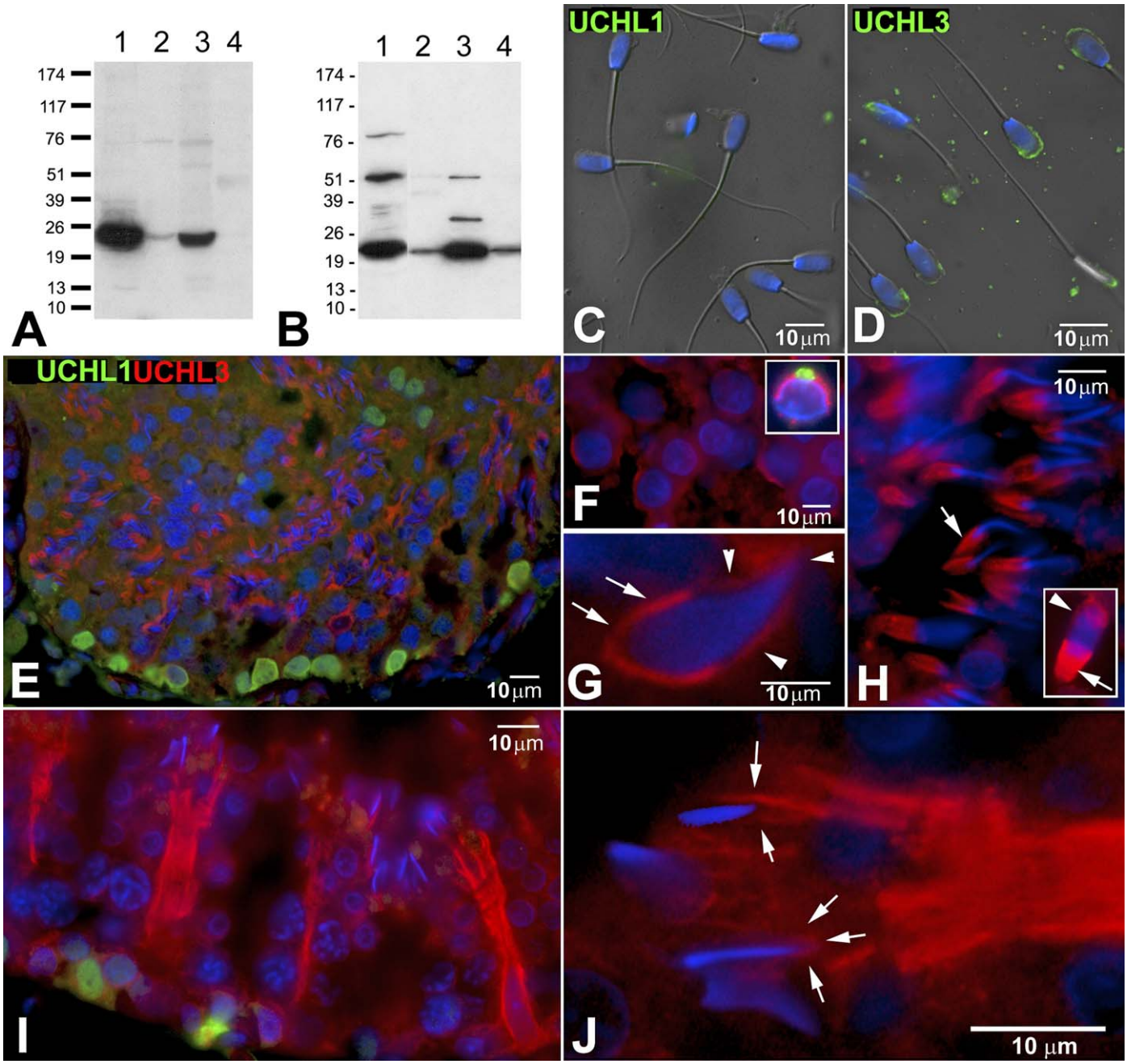

FIG. 6. UCHL3 is inserted in the boar sperm acrosome during spermiogenesis, while UCHL1 accumulates mainly in the spermatogonia. **A**) Western blotting with a monoclonal anti-UCHL1 antibody detects UCHL1 in mouse testicular cells (lane 1) and spermatozoa (lane 2), and in boar testicular cells (lane 3), but not in ejaculated boar spermatozoa (lane 4). **B**) The UCHL3 isozyme is detected in mature boar spermatozoa (lane 2), in boar testis (lane 1), and in mouse testes and spermatozoa (lanes 3 and 4, respectively). UCHL3 (**D**), but not UCHL1 (**C**), is detectable on the acrosomal surface of mature boar spermatozoa. **E**) UCHL1 (green) accumulates in the spermatogonia near the basement membrane of a boar seminiferous tubule. UCHL3 (red) is prominent in the round and elongating spermatids near the center of the tubule. **F**–**H**) UCHL3 is localized in the acrosomal cap of round spermatids (**F**), and in the manchette (arrows) and acrosomal region (arrowheads) of elongating spermatids (**G**, **H**). An acrosomal granule is labeled green with the antiubiquitin antibody and the acrosomal cap is labeled red with the anti-UCHL1/L3 antiserum (**F**, insert). **I**, **J**) UCHL3 accumulates prominently in the Sertoli cells and in the ectoplasmic specializations that form the junctions between Sertoli cells and spermatid acrosomes (arrows in panel **J**).

the inner face of the porcine oolemma is involved in the regulation of sperm incorporation and/or events leading to the establishment of the oolemma block to polyspermy, as appears to be the case in mouse.

DISCUSSION

We detected a high level of deubiquitinating activity in the boar sperm acrosome. This activity is attributable to ubiquitin-C-terminal hydrolases, such as UCHL3, present in the spermatid acrosomal cap and on the sperm acrosome. We detected and manipulated this sperm-associated UCH activity using specific UCH substrates (ubiqutin-AMC), specific UCH inhibitors (ubiquitin aldehyde), and recombinant UCHs (UCHL3 and Ipase-T). None of these reagents are cell-permeable, which suggests that the UCH activity is present on the surface of the boar sperm acrosome. The surface localization of UCH activity is supported by the high UCH

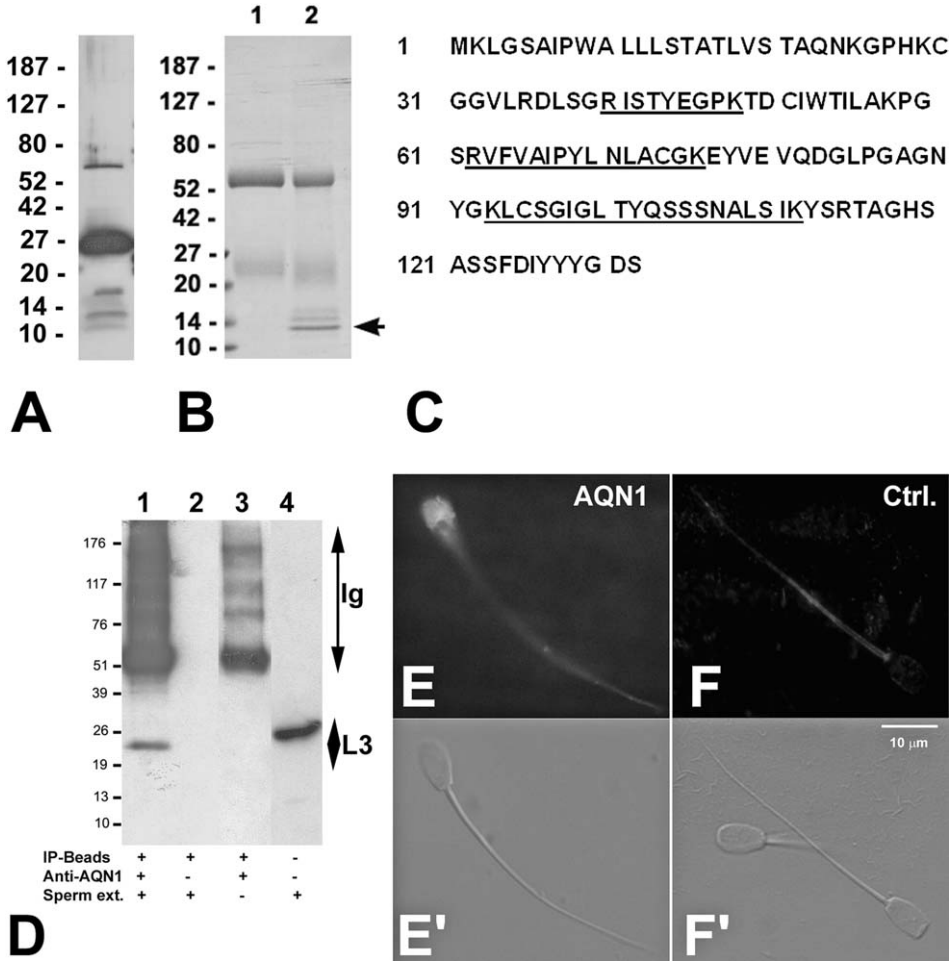

FIG. 7. Acrosomal UCHL3 interacts with spermadhesin AQN1. **A**) Anti-UCHL1/L3 antiserum recognizes the appropriate 24-kDa UCHL3 band in an immunoblot of boar sperm extracts. **B**) Coomassie blue-stained PAGE gel of proteins immunoprecipitated with the anti-UCHL1/L3 antiserum. Note the typical triplet of AQN1 bands (arrow) present in the immunoprecipitate (lane 2), but not in the control antibody only sample (lane 1). **C**) The most prominent immunoprecipitated sample is identified by MALDI-TOF (matched sequences are underlined) as porcine spermadhesin AQN1, which migrates as a typical triplet of bands in the 14–16 kDa range on SDS-PAGE. **D**) The UCHL3 band is detected by an epitope-specific anti-UCHL3 antibody in the boar sperm extracts immunoprecipitated (IP) with anti-AQN1 antiserum (lane 1). Lane 2, Sperm extract with IP beads and no antibody; lane 3, antibody and beads, but no sperm extract; lane 4, whole boar sperm extract, no IP. Ig, Rabbit immunoglobulin fragments from anti-AQN1 antiserum; L3, UCHL3 protein band coprecipitated with the anti-AQN1 antibody. **E**) Spermadhesin AQN1 is localized on the surface of the boar sperm acrosome, consistent with the immuolocalization of UCHL3. **F**) Negative control with preimmune serum to anti-AQN1 antiserum. Panels **E′** and **F′** show the corresponding DIC images.

enzymatic activity measured in the motile sperm fraction with intact acrosomes. Furthermore, the enzymatic assay of spermatozoa disrupted by sonication demonstrates that a portion of the deubiquitinating activity remains associated with the sperm head after acrosome removal, most likely through the association of acrosomal membrane vesicles with the surface of the sperm head. Consistent with the hypothesis that inhibition of deubiquitination accelerates proteasomal proteolysis [10], an increased rate of sperm penetration through the ZP was observed in the presence of UCH inhibitors and blocking anti-UCH antibodies. Furthermore, supplementation of IVF media with recombinant UCHs reduced polyspermy and increased the rate of monospermic fertilization. This polyspermy amelioration is consistent with our hypothesis that proteasomes present in the sperm acrosome recognize and degrade an ubiquitinated protein species that is present in the porcine ZP [26]. Preliminary proteomic analyses of solubilized porcine ZPs have identified this protein as *S. scrofa* ZPC (Sutovsky P, unpublished results).

Our initial biochemical analyses were performed using polyclonal antisera that recognize both UCHL1 and UCHL3. These closely related isozymes share 55% amino acid identity and due to their almost identical size, comigrate at 24 kDa in SDS-PAGE. Subtractive biochemical and immunohistochemical analyses employing epitope-specific anti-UCHL1 and anti-UCHL3 antibodies showed that UCHL3 is the UCH species present in the boar sperm acrosome. This is consistent with previous data showing that UCHL1 is mainly expressed in spermatogonia, while UCHL3 is most abundant in the spermatids of the pig and mouse [13, 15]. In porcine oocytes and zygotes, UCHL1 is the prevailing UCH isozyme in the oocyte cortex, while UCHL3 is associated with the meiotic spindle and the mitotic spindle and midbody. Accordingly, proteomic analyses of porcine [63] and bovine [64] ova have shown that UCHL1, but not UCHL3, is among the ten most-abundant oocyte proteins. UCHL1-deficient *Uchl1*^{gad} mutant mice display increased polyspermy in vivo, which has been attributed to a lack of functional UCHL1 on the oolemma [41]. According to Yanagimachi [37], the oolemma, rather than the ZP, is thought to be the primary site of antipolyspermy defense in the mouse, which explains why spermatozoa accumulate in the perivitelline spaces of mouse oocytes during IVF. In the pig, antipolyspermy defense seems to rely primarily on zona alterations by cortical granule exudates and oviductal glycoproteins [65]. Consequently, as shown in the present study, UCHL1 present in the porcine oocyte cortex does not seem to play a decisive role in antipolyspermy defense. On the contrary, manipulation of boar sperm acrosomal UCH activity alters polyspermy rates during porcine IVF. The high polyspermy rates observed in the pig IVF system could be due to the absence of limiting factors that are normally present in the in vivo ova collected after passing through oviductal fluid (i.e., oviductal glycoprotein or osteopontin) [40, 66] or to the asynchronous contribution of polyspermy-limiting factors by the spermatozoa that bind to the ZP. In the latter case, spermatozoa that first reach the ZP could deubiquitinate the surface of the ZP by their acrosomal UCH activity. Deubiquitination of the ZP surface would render the ZP unrecognizable to proteasomes of accessory spermatozoa that bind to the ZP at a later stage. In an IVF scenario, this

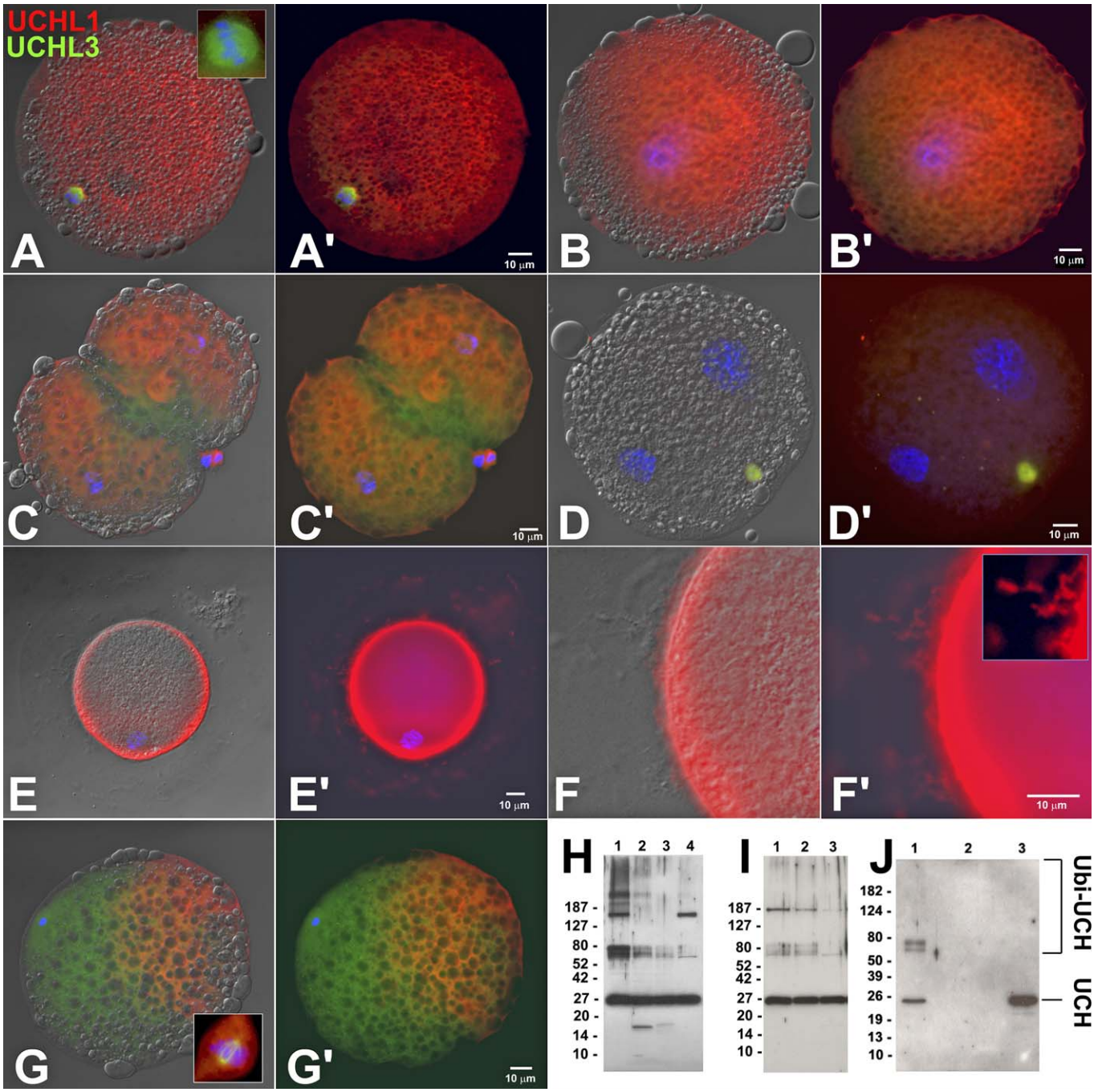

FIG. 8. **A**–**D**) Differential localizations of UCHL1 (red) and UCHL3 (green) to the cortex and meiotic spindles (insert in **A**) of porcine metaphase II ova (**A**), porcine pronuclear zygotes (**B**) and 2-cell embryos (**C**). **D**) Apart from nonspecific antibody uptake by the disintegrating polar body, no labeling is detected in porcine zygotes immunolabeled without permeabilization. **E**, **F**) In addition to cortical labeling of UCHL1, mouse ova show immunoreactivity to anti-UCHL1 antibody in granular material found in the perivitelline space after the completion of oocyte maturation (insert in **F'**). **G**, **G'**) Fusion of an enucleated porcine ovum with a fetal fibroblast that expresses both UCHL1 and UCHL3 (insert in **G**) causes polarized UCHL1 (red) distribution in the oocyte cortex without affecting the even ooplasmic distribution of UCHL3 (green). **H**–**J**) Biochemical analysis of UCH turnover after porcine oocyte activation. **H**) High-molecular-mass bands (Ubi-UCH) immunoreactive with anti-UCHL1/L3 antiserum suggest that UCHL1/L3 is ubiquitinated in MII ova. Lane 1: One hundred MII ova; lane 2: 100 zygotes cultured for 13 h after IVF; lane 3: 100 parthenogenetic ova cultured for 13 h after activation; Lane 4: 100 MII ova treated with 10 μM proteasomal inhibitor MG-132 at 6 hours after insemination. **I**) Time-lapse Western blot of UCHL1/L3 in porcine zygotes at 6 h (lane 1), 7 h (lane 2), and 8 h (lane 3) after insemination. Fifty ova were loaded per lane. **J**) Affinity purification of ubiquitinated UCHL1/L3 isoforms from porcine in vitro-matured ova using ubiquitin-binding protein p62. Lane 1, Anti-UCHL1/L3 immunoreactive bands in the ubiquitinated oocyte-protein fraction purified by p62; lane 2, control elution of p62-agarose matrix not coincubated with oocyte proteins; lane 3, whole oocyte extract (no p62 purification). Ubi-UCH, high-molecular-mass ubiquitinated UCH species; UCH, nascent, 24-kDa band.

mechanism would not be efficient, since many spermatozoa reach the ZP at the same time. Consequently, we can speculate that this is why we observe frequent polyspermy during porcine IVF and why polyspermy increases even further when we block the activities of sperm UCHs with UCH inhibitors or anti-UCH antibodies.

Taken together, our data suggest an interesting evolutionary pattern in the involvement of UCHs in antipolyspermy defense.

In the mouse, which is a mammal that relies primarily on both ZP and oolemma-residing antipolyspermy blockade [37], UCHL1 could be inserted in the oocyte plasma membrane [41] or secreted into the perivitelline space during oocyte maturation (see Supplemental Fig. 3). In humans and ungulates, which rely primarily on ZP-induced polyspermy blockade [37], the sperm-borne UCHL3 may be a primary antipolyspermic agent. In accordance with this model, proteasomal inhibitors have been shown to stop fertilization at the zona level in the pig [26] and humans [24], whereas the same inhibitors block sperm-oolemma fusion in the zona-free mouse ova [67] by altering an unknown mechanism that involves proteasomal activity.

It is possible that other deubiquitinating enzymes, including both UCHs and ubiquitin-specific proteases, are present on the sperm acrosome and contribute to antipolyspermy defense. In the future, we will investigate whether additional UCH family members contribute to the acrosomal pool. For instance, UCHL5 mRNA has been identified in mouse testicular germ cells [15], albeit not at levels comparable to those of UCHL1 and UCHL3. However, the UCHL5 molecule is substantially larger (328 AA residues) than UCHL1 or UCHL3 (223 AA and 230 AA, respectively), and the polyclonal anti-UCHL1/L3 antisera used in some of our analyses did not recognize the major 34-kDa, UCHL5-attributable band in the spermatozoa or oocytes. The functional aspect of the association of UCHL3 with the meiotic spindle will also be examined, as it could be of significance for oocyte maturation and oocyte activation at fertilization. It is well established that the ubiquitin-proteasome pathway fulfils a central role in the regulation of metaphase-anaphase transition during both mitosis and meiosis [68]. In meiosis, the association of UCHL3 with the anaphase-promoting complex could be particularly important for the sperm-induced activation of metaphase II-arrested ovulated ova.

In the pig, polyspermy defense could also be contributed by UCHs present in the oviductal fluid. This is an attractive proposition if we take into account the established role of oviductal fluid secretions in porcine antipolyspermy defense [40, 66]. The oviduct-secreted UCHs could coat oocyte and sperm surfaces during gamete transport through the oviduct and during fertilization in vivo. We detected UCHL1/L3 in isolated porcine oviductal fluid (Supplemental Fig. 6, F–H). In addition, some of the 19S proteasomal regulatory complex subunits with deubiquitinating activities, and deubiquitinating enzymes associated with these subunits, could limit the rate of sperm-ZP penetration and could be regulated by sperm physiological status (e.g., capacitation-induced changes in proteasomal subunit phosphorylation). Recently, a novel deubiquitinating enzyme that becomes activated only when associated with the 26S proteasome has been identified [69].

Several pathways could be involved in the insertion of UCHL3 into boar acrosomal membranes. Golgi-derived vesicles are thought to be the main progenitor of the spermatid acrosomal cap [70]. Newly synthesized proteins could also be transported from the spermatid cytoplasmic lobe to the acrosome along the microtubules of the caudal manchette and cytoplasmic microtubule networks at appropriate steps of spermiogenesis [71]. These pathways are consistent with the localization in the present study of UCHL3 to the acrosomal cap and caudal manchette of boar spermatids. Furthermore, several Golgi-independent pathways of acrosome biogenesis have been suggested [72], including the incorporation of endocytotic vesicles from the spermatid plasma membrane [62] and direct protein transport between Sertoli cells and sperm acrosomes via an acrosomal tubulobulbar complex that protrudes into the Sertoli cell cytoplasm [73]. The existence of such pathways opens up the possibility that the UCHL3 detected in the

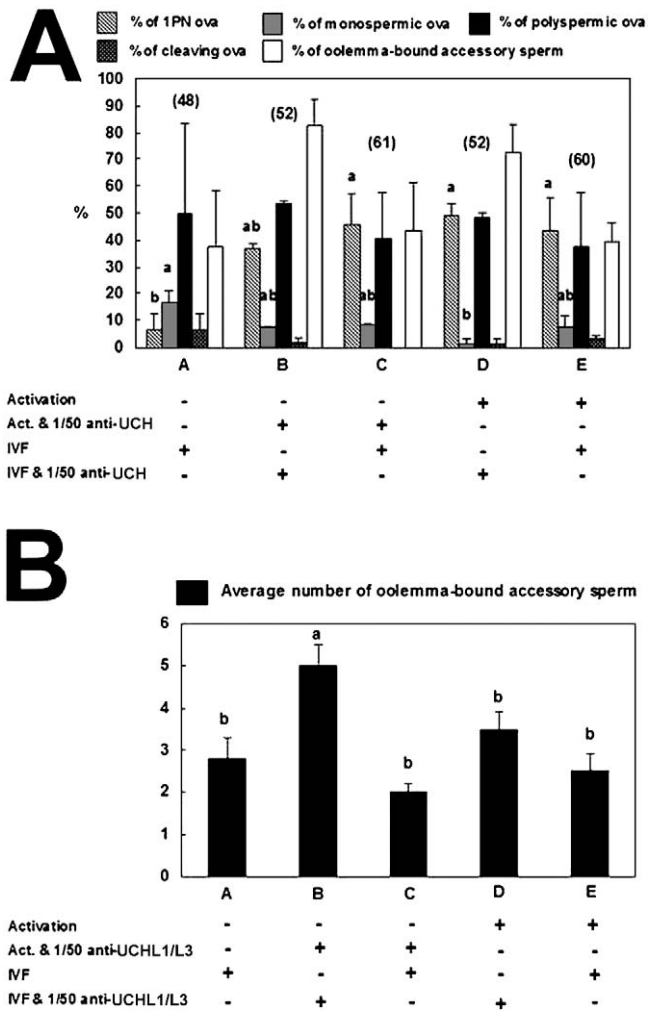

FIG. 9. Oocyte preincubation with anti-UCH antibodies has no effect on polyspermy rates during porcine IVF, with or without previous artificial activation. The values are expressed as the mean percentage ± SEM. The numbers of inseminated ova are indicated in parentheses. Superscripts a, b in each group of columns denote significant differences at $P < 0.05$. **A**) Fertilization parameters of ova parthenogenetically activated (Act.) and fertilized (IVF) in the presence or absence of rabbit anti-UCHL1/L3 antiserum (1:50 dilution). The average values from four replicates are shown. The formation of one female pronucleus is indicated by 1 PN (parthenogenetically activated ova) in the diagram. "Monospermic" ova in columns B and D are in fact parthenogenetically activated ova with two maternal pronuclei. **B**) Average number of oolemma-bound accessory spermatozoa after IVF with or without rabbit anti-UCHL1/L3 antibody (1:50 dilution) after activation (Act.) with or without anti-UCHL1/L3 (1:50 dilution).

ectoplasmic specializations that form the Sertoli cell-acrosome junctions (as shown in the present study) is absorbed onto the acrosomal surface during spermiogenesis. It will also be investigated whether UCHL3 and other UCHs can be absorbed onto the acrosomal surface during epididymal sperm maturation, as our preliminary data show the accumulation of UCHL3 in the epididymal secretory sites, the apical blebs (Supplemental Fig. 6, A–D). In this respect, Santamaria et al. [74] have found that UCH PGP9.5 (UCHL1) is secreted by rat epididymal epithelial cells and binds to the heads of epididymal spermatozoa.

Coimmunoprecipitation of UCHL3 with spermadhesin AQN1 acquired by the acrosomal surface from the seminal plasma supports the participation of UCHs in sperm acrosomal function after spermatogenesis. In addition to sperm-ZP

interactions, sperm 26S proteasomes and UCHs may be involved in sperm capacitation and detachment from the oviductal sperm reservoir. Spermadhesin AQN1 is a peripheral protein of the plasma membrane that overlies the outer acrosomal membrane. Transferred onto the sperm surface from seminal plasma after ejaculation, AQN1 has been implicated in sperm binding to the ZP [58, 75], as well as in sperm interactions with the epithelium of the oviductal sperm reservoir [61, 76]. Acrosomal AQN1 is rapidly degraded during boar sperm capacitation in vitro, coinciding with the release of spermatozoa that are bound to cultured oviductal reservoir cells [61]. Sperm proteasomes and UCHs may be involved in this rapid, regulated degradation during sperm detachment from the oviductal epithelium.

Our biochemical analysis suggests that the ubiquitinated UCHL1 protein accumulates in the ooplasm of maturing ova and is degraded by the ubiquitin-proteasome pathway upon oocyte activation by either the fertilizing spermatozoon or a parthenogenetic stimulus. A similar pattern of ubiquitination and postfertilization degradation of maternally stored proteins has been recently proposed to act in parallel with programmed degradation of maternally stored mRNAs in invertebrate eggs [77]. We have reported recently that the major vault protein (MVP), which is a cell-protecting chaperone that is thought to be responsible for drug resistance in cancer cells, accumulates in the ubiquitinated form in metaphase II porcine ova and is degraded by the 26S proteasome shortly after fertilization [57]. Similar to UCHL1, MVP is one of the most abundant proteins in the bovine oocyte proteome [63]. It is possible that abundant stores of housekeeping ooplasmic proteins are conserved in this fashion by reversible ubiquitination in the oocyte, and that their unused pool is degraded after fertilization via proteasomal proteolysis. Programmed ubiquitin-proteasome-dependent degradation of maternal proteins after fertilization may be a common developmental mechanism within the animal kingdom.

In conclusion, the modulation of UCH activity alters in a statistically significant manner polyspermy during porcine IVF, which suggests that sperm acrosomal UCHs are involved in acrosomal function and possibly in the control of sperm-ZP penetration. The modulation of UCH activities, through the use of UCH agonists or recombinant UCHs, could help in the management of polyspermy problems in mammalian IVF systems. This approach would be particularly useful for the production of developmentally competent monospermic zygotes for commercial embryo transfer.

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