ORIGINAL ARTICLE: ANDROLOGY

Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology

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Objective: To determine the levels of postacrosomal WW binding protein (PAWP) in the spermatozoa of men that were used clinically for intracytoplasmic sperm injection (ICSI) and to correlate them with infertility treatment outcomes.
Design: Prospective clinical and laboratory study.
Setting: University-based laboratory and infertility clinic.
Patient(s): Men undergoing ICSI for the treatment of couples' infertility (n = 110).
Intervention(s): Quantitative analysis of sperm PAWP levels by flow cytometry and developmental analysis of PAWP expression by immunoblotting, immunofluorescence, and immunohistochemistry.
Main Outcome Measure(s): PAWP flow-cytometric levels and immunolocalization in spermatozoa.
Result(s): A strong positive correlation was found between PAWP expression levels and fertilization rates after ICSI, with high levels of PAWP being associated with higher fertilization rates; the positive correlation was independent of age, DNA fragmentation index, and other sperm parameters. PAWP expression levels were correlated with embryonic development, with high levels of PAWP being associated with a lower number of arrested embryos within 3–5 days post-ICSI. PAWP expression was detected during the late stages of human spermiogenesis in elongating spermatids, confirming previous findings in various animal models.
Conclusion(s): Our clinical data from infertile couples demonstrate significant correlations between sperm PAWP levels and both fertilization rates and normal embryonic development after ICSI. Considering its proposed role in the initiation of oocyte activation, we suggest that PAWP could have potential applications in the diagnosis and treatment of infertility. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: Fertilization, sperm, male infertility, assisted reproductive technology (ART), intracytoplasmic sperm injection (ICSI), spermiogenesis, perinuclear theca, postacrosomal WW binding protein (PAWP)

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The intracytoplasmic sperm injection (ICSI) technique has opened a new window onto the treatment of human infertility by assisted reproductive technology (ART). Currently, ICSI is performed in approximately 67% of ART cycles in the United States; however, only 35.8% of such cycles result in clinical pregnancy (1, 2). Low or total fertilization failures

contribute to a significant percentage of those unsuccessful cycles (3–5).

Successful fertilization depends upon the activation of metaphase II arrested (MII) oocytes triggered by the fertilizing sperm. Oocyte activation involves a sequence of cellular and molecular events such as exocytosis of cortical granules, completion of meiosis II, decondensation of the sperm nucleus, pronuclei formation, and embryo cleavage. The first step of oocyte activation also involves a rapid intracellular calcium release followed by series of repetitive calcium oscillations in mammals (6–8). Although some steps of oocyte activation are well defined, it is still unclear which sperm-borne oocyte activating factor(s) (SOAF) are required for initiating intracellular calcium release and embryo development.

We have introduced postacrosomal WW binding protein (PAWP, also known as WBP2NL) as a major SOAF candidate protein (9–11). Microinjection of recombinant PAWP protein into MII oocytes of *Xenopus* and mammals induced a high rate of meiotic resumption and pronuclear formation. Furthermore, the sperm-induced fertilization was successfully blocked by corresponding antibodies against PAWP or competitive peptides derived from its sequence, suggesting PAWP as a strong SOAF contender (9, 10). Other observations on the mouse and rat have also demonstrated that PAWP is assembled during spermiogenesis in the postacrosomal sheath of perinuclear theca (PAS-PT) (11). PAS-PT is a nonionic detergent-resistant component of the sperm head whose content is the first to be dispersed into the oocyte cytoplasm after sperm-egg fusion at fertilization (12, 13). Unlike the other SOAF candidate, phospholipase Cζ (PLCζ), PAWP is retained by the sperm on entry into the oocyte cytoplasm during fertilization (10, 14).

Considering failure of oocyte activation as a major cause of fertilization failure in couples undergoing ART and the proposed role of PAWP in facilitating this signaling process, the purpose of this prospective study was to investigate whether sperm levels of PAWP may play a vital role in the success of infertility treatment. To this end, we screened human sperm samples used for ICSI and found strong correlations between PAWP expression and fertilization success as well as normal embryonic development.

MATERIALS AND METHODS

Human materials for the study were donated by men undergoing IVF or ICSI procedures at the CReATe Fertility Centre, Toronto, Ontario. Written informed consent was obtained from all participants, and the study was approved by the Research Ethics Board of the Sunnybrook Health Sciences Centre, Toronto, Canada (Project ID no. 076-2012) as well as by the Health Sciences Research Ethics Board of the University of Toronto (Protocol Reference no. 28888). Unless otherwise mentioned, all chemicals used were purchased from Sigma Chemical Co.

Antibodies

Polyclonal rabbit serum was raised against human recombinant (r) PAWP protein that was produced from a PAWP-cDNA expression vector (pET28a-N-His-Tag) within *E. coli* host BL21 (DE3). The serum was then affinity purified on immobilized His-tag purified rPAWP protein and eluted with glycine followed by neutralization with Tris-buffered saline (pH 8). For PLCζ, polyclonal rabbit antiserum against two peptides of human PLCζ (C-RESKSYFNPSNIKE-coNH_2; CE-THERKGSDKRGDN-coNH_2) from Covalab was used (14, 15).

Immunoblotting

Immunoblotting was used to validate the activity of PAWP antibodies and to verify PAWP expression in human spermatozoa. Also PLCζ antibodies were used to verify their localization to the sperm membranous and acrosomal structures. Human sperm samples were dissolved in reducing sample buffer containing 2% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol. Equal amounts of sperm extracts (1×10^7 sperm/lane) were loaded and resolved on 10%–12% SDS–polyacrylamide gel electrophoresis. Proteins were transferred to either nitrocellulose (Schleicher and Schuell) or polyvinylidene fluoride (Millipore) membranes. Affinity-purified anti-human PAWP antibody (1:200) or anti-human PLCζ antibody (1:1000) were diluted in phosphate-buffered saline (PBS) containing 0.05% Tween and used for incubation with the membranes for 3 hours at room temperature or overnight at 4°C. The immunoreactivity on Western blots was detected with peroxidase labeled goat anti-rabbit IgG (Vector Laboratories Inc.), diluted 1:25,000 (v/v), and further use of enhanced chemiluminescent substrate (Pierce) with exposure to X-ray films.

Immunoperoxidase Staining for Light Microscopy

The paraffin-embedded human testicular tissues (n = 5), obtained from known fertile cancer patients who underwent total orchiectomy in Kingston General Hospital, Queen's University, were processed for immunoperoxidase staining according to previously described protocols (14, 16). Slides were incubated with anti-human PAWP antibody (1:50) overnight at 4°C and then washed and further incubated with biotinylated goat anti-rabbit IgG as the secondary antibody (1:200, Vector Labs) for 1 hour at room temperature. The processed sections were counterstained after washing with 0.1% methylene blue and visualized by Nikon Eclipse E800 microscope.

Sperm Samples and Preparation for PAWP Expression Analysis

Surplus sperm after use clinically for ICSI was collected for analysis. Semen samples were collected at the day of the oocyte retrieval by masturbation on site after a 2- to 5-day period of sexual abstinence. The semen was allowed to liquefy and analyzed within 60 minutes of collection. Routine semen analysis was performed with evaluation of semen volume, sperm concentration, sperm motility, and sperm morphology according to World Health Organization recommendations (17). Sperm DNA damage was assessed by flow cytometry as described elsewhere (18). For each sample, 5,000 cells were analyzed on two aliquots with a