## UBIQUITINATION OF RETAINED DISTAL CYTOPLASMIC DROPLETS ON EJACULATED PORCINE SPERMATOZOA REVEALED BY IFA *IN SITU*

Christopher E. Kuster[1], Rex A. Hess[2], Gary C. Althouse[3]

[1]Dept. of Veterinary Clinical Medicine, University of Illinois, Urbana, IL, USA
[2]Dept. of Veterinary Biosciences, University of Illinois, Urbana, IL, USA
[3]Dept of Clinical Studies - NBC, University of Pennsylvania, Kennett Square, PA, USA

Specific sperm organelles (e.g., mitochondria) are tagged by ubiquitin, a universal proteolytic marker, during spermatogenesis and are subsequently degraded by oocyte lysosomes after fertilization, while ubiquitin-free organelles (e.g., centrosome, male pronucleus) are critical to normal zygotic development. It has been proposed that ubiquitin present on the surface of sperm from subfertile ejaculates may be carried over to the oocyte cytoplasm, where it could then target vital paternal organelles for destruction and prevent further embryonic development. The internal membranes of the boar sperm cytoplasmic droplet (CD) are estimated to comprise greater than 54% of the total surface area of the sperm cell, and, if ubiquitinated, could contribute significant amounts of ubiquitin to the developing zygote. The objectives of this study were to I) develop a technique to optimize distal cytoplasmic droplet (DD) retention *in situ* for immunostaining, and II) determine if ubiquitin-specific antibodies recognize antigens associated with boar sperm CD. Formalin is the fixative of choice for immunochemistry, but was found in our study to be ineffective at maintaining DD *in situ* during processing. Substitution with glutaraldehyde (0.2%), however, yielded a high mean retention rate of 88% for DD on ejaculated boar sperm, while 2% neutral buffered formalin and PBS controls resulted in only 29% and 20% retention rates, respectively. This new technique maintained DD *in situ* on ejaculated porcine spermatozoa throughout the process of permeabilization and immunostaining. Additionally, conducting all fixation and incubation steps in microtubes provided clearer image resolution with a more uniform, higher density sperm cell distribution compared to the more commonly used pre-mounting technique whereby sperm cells are attached to coverslips or charged glass slides prior to fixation and processing. Following permeabilization with the detergent TX-100 (0.05%), ubiquitin staining was easily observed in nearly all CD. Immunoblotting indicated a greater content of ubiquitinated proteins present in extracts from sperm cells from an ejaculate with an abnormally high percentage of retained DD (52% DD) compared to a morphologically normal sample (<10% CD). Western blot demonstrated that the primary antibody recognized both mono-ubiquitin of bovine origin (8.5 kDa), and human ubiquitin conjugate (35 kDa). Since superfluous ubiquitin species may negatively impact embryonic development, the presence of antigens recognized by anti-ubiquitin antibodies in the boar sperm CD raises concerns that retained DD may be more detrimental to fertility in litter-bearing species than previously suspected.

Key Words: boar, cytoplasmic droplet, sperm, ubiquitin