# Sperm acrosome associated 3 protein expression in equine primordial, primary, secondary, and tertiary follicles

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## Abstract

The objective of this study was to characterize the protein expression of sperm acrosome associated 3 (SPACA3) in the equine ovary. Formalin-fixed and paraffin-embedded ovarian sections from 16 horses were processed for routine immunohistochemistry for SPACA3. Representative images were digitally captured at 400 x magnification. In all mares, SPACA3 was expressed in granulosa cells of all stages of follicles. Expression of SPACA3 in all equine follicular stages suggests that this may be a permanent immunosterilant target for the management of feral horse herds. Additional research is needed to determine if horses can produce a robust humoral response to a SPACA3 vaccine to induce sustained infertility.

**Keywords**: Granulosa cell, horse, ovary, sperm acrosome associated 3 protein

## Introduction

Sperm acrosome associated 3 (SPACA3, also known as sperm protein reactive with antisperm antibody (SPRASA) and sperm lysosome-like protein 1 (SLLP1), is a unique, intra-acrosomal, nonbacteriolytic, conventional-type lysozyme-like protein of mammalian sperm.[1,2] Sperm acrosome associated [3] is expressed on the inner acrosomal membrane of human, cattle, sheep, and deer sperm.[3] In addition to other testis specific lysozyme-like proteins, SPACA3 has been identified as a biomarker for male fertility.[4-7] Sperm acrosome associated 3 is also reported to have a role in sperm-egg plasma membrane adhesion and fusion during fertilization in humans1 and mice.[8]

Sperm acrosome associated 3 might have a role in female reproduction. Women experiencing infertility have higher concentrations of SPACA3 antibodies than fertile women.[9] Additionally, female mice immunized against SPACA3 had profound infertility.[9] In cattle, dogs, and cats, SPACA3 is expressed in ovarian follicles at all stages of development and localized to the ooplasm and granulosa cells with weak staining in theca cells.[9] In a preliminary study, SPACA3 expression was examined in the equine ovary of 3 horses.[10] The objective of the current study was to confirm SPACA3 expression in the equine ovary from a larger sample size consisting of both domesticated and feral mares

## Materials and methods

Both domesticated mares (n = 8) and feral mares (n = 8) were used in this study. Domesticated mares (3 - 14 years old) were pastured on a ranch located in Stayton, Oregon. Each pasture contained a 4.9 x 7.3 meter, 3-sided shelter. Feral mares (3 years old) were maintained at the Warm Springs herd management area located in Harney county, Oregon. The procedures were conducted under a protocol approved by the institutional animal care and use committee of Oregon State University (protocol #3924).

Ovaries were obtained via a standing colpotomy performed by an experienced veterinarian. Briefly, feed was withheld for 36 hours prior to surgery. For sedation and analgesia, detomidine hydrochloride (0.02 - 0.04 mg/kg), butorphanol tartrate (0.01 - 0.04 mg/kg), and xylazine hydrochloride (0.9 - 1.2 mg/kg) were given intravenously. Surgical preparation included wrapping the tail with gauze and tying it up to keep it out of the surgical field, transrectal palpation to manually evacuate the feces, scrubbing the perineal area with chlorhexidine (Vet Solutions, Inc., Bedford, TX), and flushing the vagina with very dilute povidone iodine. An incision in the anterior dorsolateral wall of the vagina was made, 2% lidocaine was injected into each ovarian pedicle for local analgesia, and the ovaries were removed using a chain ecrasure. The vaginal incision was left to heal by second intention. For postsurgical analgesia, flunixin meglumine was given intravenously (1.2 -1.5 mg/kg) and buprenorphine hydrochloride (10 mg) was given subcutaneously. Additionally, each mare received intramuscularly the long-acting antibiotic ceftiofur crystalline-free acid (6.6 mg/kg).

Ovaries were hemi-sectioned, fixed in 10% neutral buffered formalin, and paraffin embedded. Sections were serially sectioned (4 µm) on charged slides for routine immunohistochemistry for SPACA3. Slides from all mares were processed in 1 experiment

to avoid variations. Briefly, slides were deparaffinized in xylene and rehydrated in a graded ethanol series (100, 75, and 50%). Antigen retrieval was conducted by incubating sections in a citrate buffer, Target Retrieval Solution #S1699 (Dako North America Inc., Carpinteria, CA) in a microwave for 10 minutes and cooled for 20 minutes. Slides were then washed in buffer (Dako North America Inc., Wash buffer #S3006) and tissue-specific endogenous peroxidases were inhibited by incubating slides in 3% hydrogen peroxide. Slides were washed again and nonspecific binding was blocked with serum-free protein (Protein block serum-free ready to use, #X0909, Dako North America Inc.) for 20 minutes at room temperature. Subsequently, slides were tapped off and the primary antibody (#21137-1-AP, Proteintech, Rosemont, CA) diluted 1:200 (background reducing components, #S3022, Dako North America Inc.) was applied to sections for 105 minutes at room temperature. A fusion protein with the following sequence was used to produce primary antibodyPYAGVCLAYFTSGFNAAALDYEADGSTNN-GIFQINSRRWCSNLTPNVPNVCRMYCSDLLNPNLKDTVI-CAMKITQEPQGLGYWEAWRHHCQGKDLTEWVDGCDF. Primary antibody reactivity was confirmed by the manufacturer in the mouse testis and was also confirmed in a preliminary experiment in the equine testis. Specificity of immunostaining was verified by replacing the primary antibody with negative control rabbit serum (negative control rabbit IgG, #NC495H, Biocare Medical, Pacheco, CA). Slides were washed in buffer and a secondary antibody (One step horseradish peroxidase-conjugated polymer antirabbit IgG, #IH-8064-OSU-15, Immuno BiosCience, Mukilteo, WA) was applied to sections for 30 minutes at room temperature. The secondary antibody was washed off and NovaRED (#SK4800, Vector Laboratories Inc., Burlingame, CA) was applied to sections for 5 minutes at room temperature. Sections were then counter stained in hematoxylin, dehydrated in a graded series of ethanol (50, 75, and 100%), moved through a series of 3 xylene baths and cover slipped.

Slides were evaluated by a single observer at 400 x magnification with a bright-field microscope (Leica DM4000B, Leica Microsystems Inc. Buffalo Grove, IL). Representative images from each ovary were electronically captured using a digital camera (QImaging, QICAM 12-BIT, #QIC-F-M-12-C, Surrey, BC, Canada) and image capture software (QCapturePro, Surrey). The cellular expression on SPACA3 was recorded for primordial, primary, secondary, and tertiary follicles.

## Results

SPACA3 was localized (Figure 1) to the sperm acrosomes in the equine testis, and to the pregranulosa cells of primordial follicles (Figure 2), and to the granulosa cells of primary (Figure 2A), secondary (Figure 2B) and tertiary follicles (Figure 2C) of all equine ovaries examined. There was no positive staining in any other cell type. In addition, slides stained with the universal negative did not have any positive staining (Figures 1 and 2).

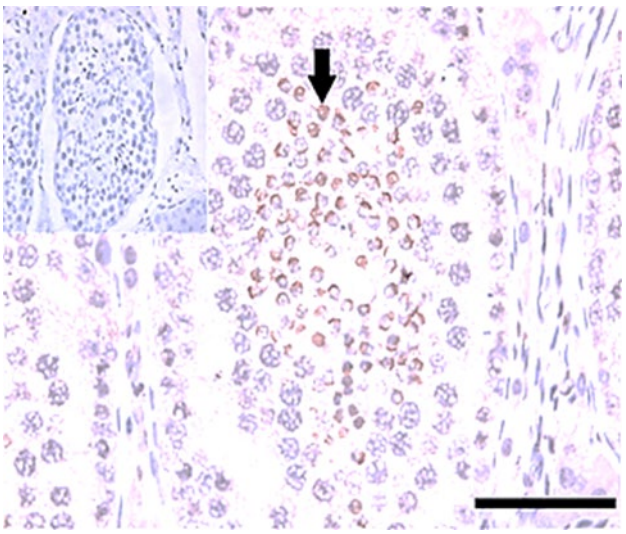

**Figure 1**. SPACA3 immunoexpression (arrow) in the equine testis is localized to the sperm acrosome (scale bar = 20 µm). Negative control in upper left inset.

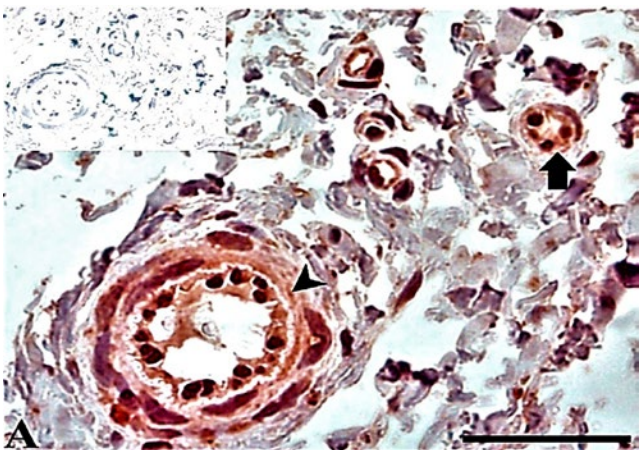


**Figure 2A.** SPACA3 immunoexpression in equine ovarian follicles. A: pregranulosa cells in primordial follicles (arrow) and granulosa cells in primary follicles (arrowhead) express SPACA3 (scale bar = 25 µm).

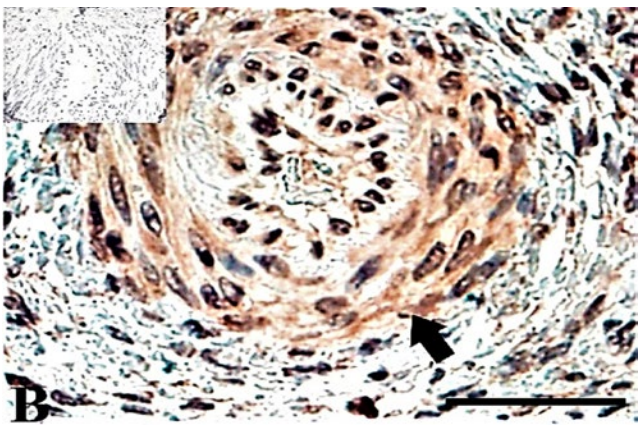


**Figure 2B.** SPACA3 immunoexpression in equine ovarian follicles. granulosa cells in secondary follicles (arrow) express SPACA3 (scale bar = 100 µm).

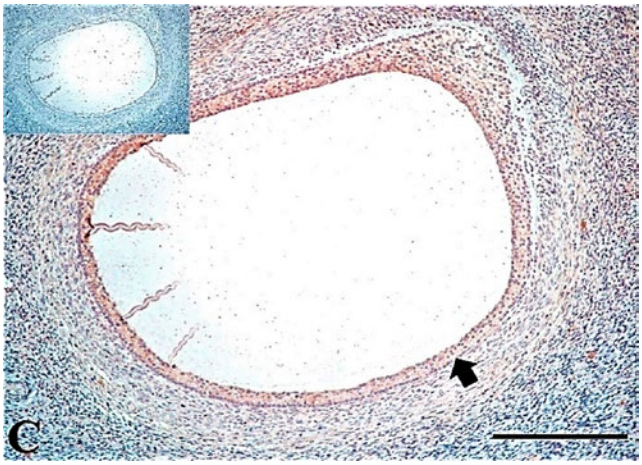


**Figure 2C.** SPACA3 immunoexpression in equine ovarian follicles. granulosa cells in tertiary follicles (arrow) express SPACA3 (scale bar = 350 µm). Negative controls in upper left insets

## Discussion

As in cattle, dogs, and cats, SPACA3 was present in equine pregranulosa cells of primordial follicles and granulosa cells of primary, secondary, and tertiary follicles. However, unlike in dogs and cats, the equine oolemma did not appear to express SPACA3. The equine oolemma appears to have a lower capacity for spermatozoa-oolemma fusion and penetration rates compared to other species that could explain the lack of SPACA3 expression in horses.[11]

In the US, the feral horse population has drastically exceeded the carrying capacity of the public lands where they are managed.[12] Immunization of horses against porcine zona pellucida (PZP) results in an antibody-based block to sperm-oocyte binding and subsequent fertilization, with a variable efficacy of immunocontraception.[13-17] Current PZP vaccines have a duration of efficacy for up to 2 years and need to be given again to provide continued immunocontraception.[14,17-18] Because oocytes in the primordial follicles lack a zona pellucida, the ovarian reserve follicles remains unaffected by PZP vaccines. Gonadotropin releasing hormone (GnRH) vaccines have been used in horses. Vaccines against GnRH stimulate antiGnRH antibody production that inhibit the release of FSH and LH and therefore prevent estrous cyclicity.[19] Similar to PZP, as the GnRH antibody titers decline over time, normal estrous cyclicity returns in mares.

This work has provided a foundation for future research in the development of a permanent immunosterilant for the management of feral horse herds. A permanent nonsurgical sterilization method for feral horses is highly desired by public land managers. An ideal immunocontraceptive for feral horses would target primordial follicles and developing follicles. SPACA3 is strongly expressed in the pregranulosa cells of equine primordial follicles; therefore, development of a SPACA3 vaccine for horses might result in permanent sterilization. Additional research is needed to determine if horses can produce a robust humoral response to a SPACA3 vaccine to in order to induce sustained infertility.

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## Conflict of interest

Authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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