Validation of Antibodies for the Immunolocalization of Germ Cells in Alligator Gonads.

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Methods

1. Tissue Processing
Hatching alligator gonad-adrenal-mesonephric kidney (GAM) complexes were formalin fixed, dehydrated, cleared, and imbedded in paraffin wax. GAM complexes were sagitally sectioned at 5 µm and mounted on positively charged slides.

2. Immunohistochemistry: Antigen Retrieval and Blocking
Slides were deparaffinized and rehydrated prior to antigen retrieval, during which slides were immersed in citrate buffer (pH 6.0) under pressure at 110ºC for 20 minutes. Blocking (Fig. 2) was performed by incubating sections in Tris-buffered saline (TBS) containing 0.25% Triton X-100, 1% bovine serum albumin (BSA), and 10% normal goat serum (NGS) for 1 hour at room temperature.

3. Immunohistochemistry: Primary and Secondary Antibodies
Primary antibody was diluted in TBS with 0.02% Triton X-100, 1% BSA, and 10% NGS prior to overnight incubation at 4ºC.

Prior to incubation with secondary antibodies conjugated with horseradish peroxidase (HRP), tissue sections were treated with 0.3% H2O2 to reduce endogenous peroxidase activity. Following a 1-hour incubation with HRP-conjugated antibody, slides were developed with the addition of 3,3’-diaminobenzidine (DAB). Hematoxylin counterstaining was performed prior to coverslipping. Slides incubated with secondary antibodies conjugated with Alexa Fluor 488 were coverslipped with VectaShield mounting medium and allowed to cure for 48 hours prior to sealing.

Results

Positive control mouse testes stained with DDX4 primary and HRP conjugated secondary antibodies showed staining specific to germ cells. Staining of alligator GAM appeared specific to putative germ cells in the ovarian cortex and seminiferous tubules (Fig. 3).

Detection with fluorophore-conjugated secondary antibody in mouse testes was specific to cytoplasmic staining of spermatagonia and spermatocytes. Staining in alligator GAMs was not specific to putative germ cells in the ovarian cortex or seminiferous tubules (Fig. 4).

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Conclusions

The IHC protocol used for the immunolocalization of germ cells expressing SCP3 and DDX4 worked as expected in the positive control mouse tissue. Our initial results using HRP-conjugated secondary antibodies suggested that the monoclonal antibody to SCP3 did not recognize homologous antigens in alligator (results not shown), whereas staining with the polyclonal antibody DDX4 was most intense in the region of putative germ cells in neonatal alligator gonads. Further testing with fluorophore-conjugated secondary antibody contradicted those results, with high background staining throughout the GAM, and the most intense staining in adrenal tissue. Background staining in sections incubated with secondary antibody only was negligible, suggesting that our primary antibody is responsible for most of the non-specific binding. We believe the discrepancy is due to the lower resolution offered with the HRP-DAB system.

Our results underscore the challenges of IHC, particularly in species for which antibodies are not readily available. The ability to immunolocalize germ cells in alligators will aid in our understanding of the effects of environmental contaminant exposure; therefore, we will continue test candidate antibodies for this purpose.

References


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