Analysis of Anti-Müllerian Hormone Expression in the Ovary: Exploring Modified Immunochemistry Protocol for Laboratory Learning

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Abstract

Background: Immunohistochemistry (IHC) is a widely used laboratory method that combines anatomical, immunological, and biochemical techniques to detect the presence of specific antigens in tissues. Although IHC is the most general application of immunostaining, it has not been commonly introduced in undergraduate courses due to the prolonged and complex experimental protocol. Therefore, experiments were designed to develop an IHC protocol that is more achievable in an educational laboratory setting. **Methods:** Specifically, IHC was performed to detect anti-Mullerian Hormone (AMH) expression. In female mammals, AMH is mainly produced by granulosa cells in the ovary and is involved in regulating follicle growth to produce mature oocytes for fertilization.[4] The hypothesis was that an IHC protocol with modified antibody concentration and incubation period could yield specific AMH staining in granulosa cells. Formalin-fixed paraffin-embedded mouse ovary sections were used for IHC staining. The rabbit anti-mouse AMH polyclonal antibody underwent 1:1000, 1:2000, 1:4000, and 1:8000 dilutions. The antibody incubation period included 1, 2, 3, 4, 5, 6, and 7 days. Sections incubated with antibody diluent served as the negative control. Experiments were conducted in duplicates. **Results:** The results showed that AMH staining could be detected primarily in granulosa cells, but not other ovarian cells, using the modified IHC protocol. The non-specific background staining was observed with increased antibody concentration and incubation time. The relatively high contrast images were obtained utilizing 1:1000 antibody dilution and 2-day incubation or 1:8000 antibody dilution and 7-day incubation. AMH staining was not detected in the negative control sections. **Conclusion:** The specific AMH staining detected using the modified protocol suggests that IHC can be successfully performed by undergraduate students for laboratory learning. Future studies will further improve the protocol by reducing background staining. The optimized IHC protocol can be useful for undergraduate laboratory classes, including but not limited to histology, immunology, biochemistry, and embryology.

Introduction

Immunohistochemistry (IHC) is a technique employed to identify antigens within cells of a tissue section by leveraging the interaction between antibodies and specific antigens in biological tissues. The resultant binding of antibodies to antigens is visualized through various means with commonly used enzyme like Streptavidin Peroxidase (SP) or other catalyzing color-producing reactions.[4] Widely adopted in research and clinical laboratories, IHC allows for the visualization of specific cellular components in their proper tissue context, offering insights into distribution and localization. In this research, IHC staining was used for the localization of Anti-Müllerian hormone (AMH) (Figure 1). Anti-Müllerian hormone (AMH) is produced exclusively by granulosa cells in females. AMH production increases from primordial to preantral follicle stage and is highest in preantral and small antral follicles. It is a peptide hormone produced by granulosa cells in the preantral and early antral follicles. AMH secretion begins when follicles differentiate from the primordial to the primary stage, peaks during preantral and antral stages, and declines when the follicle has reached the midantral stage.

Methods

The mouse ovarian tissue samples underwent standard staining procedures involving Immunohistochemistry (IHC) and Hematoxylin counterstaining. IHC staining involves the use of antibodies that bind selectively to the target protein or antigen of interest, Anti-Mullerian Hormone (AMH). By labeling the target protein with a enzyme-linked secondary antibody, biotinylated goat anti-rabbit (IgG), researchers can observe its presence and distribution within the tissue sample under a microscope. In IHC, Hematoxylin served as a counterstain to enhance contrast for specific immunostaining, imparting a blue color to cell nuclei. This contrasted with the color of the immunohistochemical reaction product, aiding in the visualization and interpretation of the staining patterns within the tissue samples. The rabbit anti-mouse AMH polyclonal antibody underwent 1:1000, 1:2000, 1:4000, and 1:8000 dilutions. The antibody incubation period included 1, 2, 3, 4, 5, 6, and 7 days.

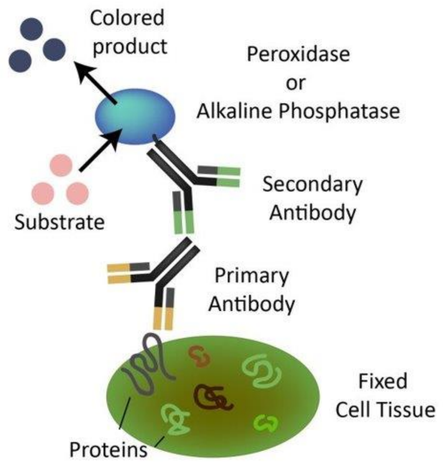

Figure 1. **Immunohistochemistry (IHC) Staining Theory [5] :** Illustration depicting the principles of Immunohistochemistry for visualizing the presence and localization of proteins in tissue sections. The process involves the use of primary antibodies, such as the specific Anti-Müllerian Hormone (AMH) antibody in this study, to target markers associated with preantral follicles. Secondary antibody selection involved biotinylated goat anti-rabbit IgG (H+L), enhancing the detection specificity. Streptavidin Peroxidase was employed as the visualization reagent due to its unique property as a biotin-binding protein, minimizing non-specific binding events. Streptavidin Peroxidase offers robust and precise visualization, crucial for accurate interpretation of immunohistochemical results.

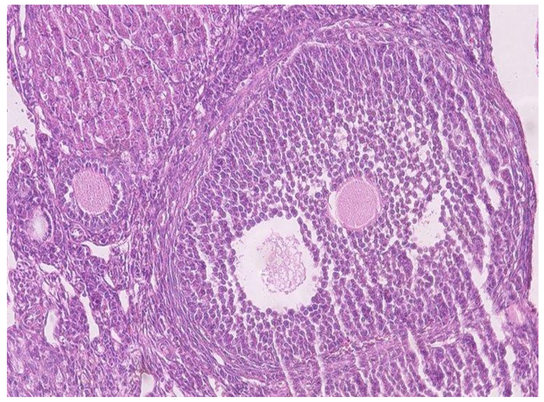

Figure 2. Standardized H&E staining of Large and Small Preantral follicles in mouse ovary tissue sample.

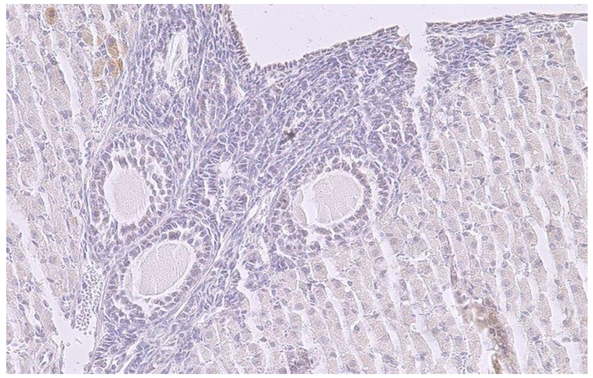

Figure 3. **1:000, 1 day IHC Negative Control;** Stained section without the primary antibody;This figure highlights the necessity of proper negative controls in IHC to validate interpretations. Omitting the primary antibody while maintaining other steps allows verification that observed reactions result specifically from the interaction between the epitope and secondary antibody. Emphasizing that controls solely omitting the primary antibody are inadequate, this control design ensures reliable IHC results by ruling out nonspecific bindings.

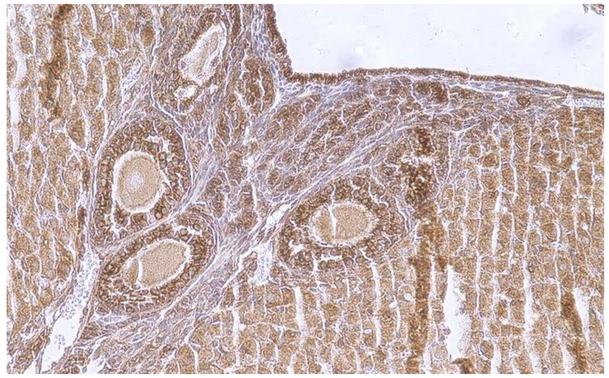

Figure 4. **1:1000 dilution, 1 day;** An image of AMH-positive small preantral follicles, granulosa cells, and theca cells surrounding the oocyte. These cells were stained using standard IHC staining protocol. Dark brown coloration indicates the presence of AMH, seen in granulosa cells surrounding the oocyte

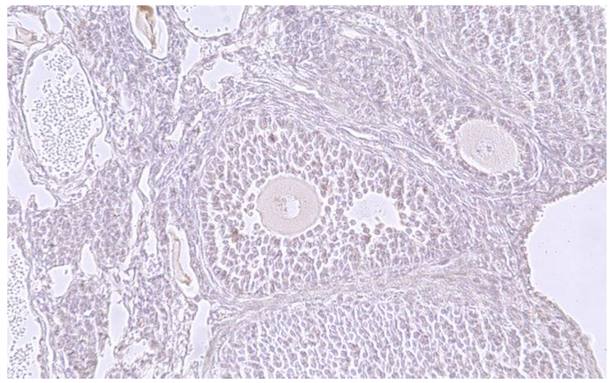

Figure 5. 1:4000 , 7d IHC Negative Control. This image shows there was no non-specific binding within the tissue sample. The absence of brown staining means the secondary antibody did not bind to the epitope (AMH) in this sample. This indicates the necessity of the primary antibody for successful IHC staining as an identification technique for specific hormones. At 7 days and 1:4000 concentration

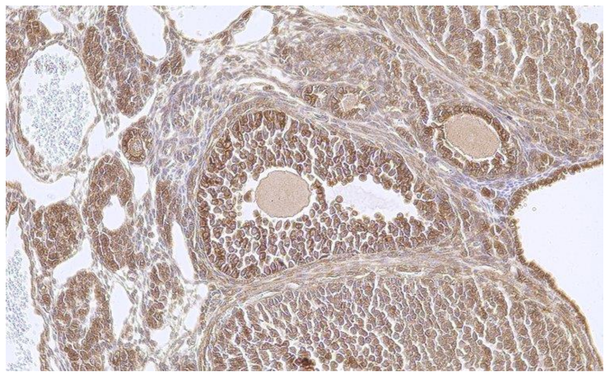

Figure 6. 1:4000 dilution, 7 day; An image of AMH-positive large preantral follicle, granulosa cells, and theca cell surrounding the oocyte. Brown staining indicates the presence of AMH in the cells. Staining with our improved protocol after 7 days, shows to be successful. Image compares to standard procedure seen in *Figure 4*. Quality of both images is similar, supporting success in the improved protocol.

Results and Conclusion

Results: The investigation into AMH staining in ovarian tissue using a modified IHC protocol yielded significant findings. The staining was predominantly localized in granulosa cells, shedding light on the specific cellular distribution of AMH within the ovary. However, caution is needed to avoid non-specific background staining, which was observed with increased antibody concentration and incubation time. The study identified optimal conditions for achieving high-contrast images, notably a 1:1000 antibody dilution with a 2-day incubation period and a 1:4000 antibody dilution with a 7-day incubation period. Importantly, negative control sections did not exhibit AMH staining, confirming the specificity of the protocol. The results culminated in an updated protocol suitable for undergraduate laboratories, balancing practicality and reliability. The 1:4000 antibody dilution with a 7-day incubation period emerged as a successful approach, offering a valuable resource for educational settings and contributing to our understanding of AMH in ovarian tissue. **Conclusions:** Our modified IHC protocol demonstrates the feasibility of incorporating specific AMH staining into undergraduate laboratory learning. To ensure reliable results, additional steps outside regular lab hours are required, necessitating mentorship or oversight by experienced individuals. Overall, this study highlights the potential of undergraduate students in performing IHC, emphasizing the importance of mentorship for successful execution and offering a valuable educational experience across disciplines. By enhancing and refining IHC protocols, we have paved the way for more accurate and reliable localization of specific antigens within tissues.

Future Work

Our research in immunohistochemistry (IHC) holds promising implications for future work in the field. Our findings may inspire further innovation in IHC techniques, leading to the discovery of novel biomarkers and therapeutic targets for various diseases. Overall, our efforts in optimizing IHC protocols have the potential to significantly impact future research endeavors, driving advancements in both basic science and clinical applications. To ensure reliable results, additional steps outside regular lab hours are required, necessitating mentorship or oversight by experienced individuals. Moving forward, further refinement of the protocol aims to minimize background staining, enhancing the reliability and accessibility of the IHC technique for undergraduate classes.

References and Acknowledgments

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We would like to give special thanks to the BIOL 403-001 and 002 students of Fall 2023 who contributed to our research through experimentation, to Dr. Yingguang Liu from LUCOM, who graciously assisted us in using the KEYENCE BZ-X800 Series All-in-One Fluorescence Microscope for clearer photos of our tissues, and to The Department of Biology and Chemistry for printing out this poster.