

Study Protocol

Comparison of Microwave Treatment and Pressure Cooker Methods for Antigen Retrieval Techniques In Immunohistochemistry

ABSTRACT:

BACKGROUND: Immunohistochemistry (IHC) is a method which is capable of detecting antigens or antibodies on the cells. The antigen antibody complex formed as a result of this reaction can be visualized at a light microscopic level. The main procedure involves application of a primary antibody which is targeted against a selected tissue antigen. Immunohistochemistry implements various polyclonal and monoclonal antibodies which help to identify the tissue location of the disease. Antigen retrieval (AR) is a key step in IHC which can be done by two methods: 1] Pressure cooker treatment 2] Microwave treatment method. This study aims to compare antigen retrieval efficacy and quality of immunohistochemical sections obtained by these two methods.

METHODOLOGY: This prospective analytical study will be conducted in the histopathology and immunohistochemistry divisions of department of Pathology, J.N.M.C. Wardha. The study will evaluate immunohistochemical sections during which antigen retrieval would be done either by microwave treatment method or autoclave method in 30 cases. All the cases will be divided in two groups. For Group 1 - antigen retrieval will be performed by microwave treatment method. For Group 2 - antigen retrieval will be done by autoclave method. Qualitative evaluation and comparison of the sections obtained by these 2 methods will be done. Statistical evaluation will be done by SPSS software version 22.0, Prism 6.0 analysis software. Chi square test will be used for comparison.

RESULTS: A significant difference is expected in the between the efficacy of antigen retrieval by pressure cooker method and microwave treatment.

CONCLUSION: Conclusions will be drawn on careful analysis of results.

KEYWORDS: Pressure Cooker Antigen Retrieval Method, Microwave Treatment Method. Antigen, Antibody, Immunohistochemistry, Histopathology.

INTRODUCTION:

IMMUNOHISTOCHEMISTRY AND IMMUNOHISTOCHEMICAL STAINS:

Immunohistochemistry (IHC) is a method which is capable of detecting antigens or antibodies on the cells. [1]The antigen antibody complex formed as a result of this reaction can be visualized at a light microscopic level. The procedure was conceptualized and first implemented by Albert Coons in 1941.[2]The main procedure involves application of a primary antibody which is targeted against a selected tissue antigen. Immunohistochemistry

implements various polyclonal and monoclonal antibodies which help to identify the tissue location of the disease.[3]

Immunohistochemistry is widely used for the sub-typing of various malignancies because certain antigens are abnormally expressed in certain cancers.[4]IHC utilizes biopsies, which are then processed further into sections. After this, the sections are incubated with primary and/ or secondary antibodies.[5]The antigen antibody complexes which are formed as a result of the reaction get localized either in the cytoplasm, nucleus or the cell membrane. The location where this reaction takes place (nucleus, cytoplasm and membrane) has a wide range of clinical implications in the management of various disorders. Special reagents like fluorescein and colloidal gold are the various markers used for visualization of these antigen antibody reactions.[6]

The basic steps in Immunohistochemistry are as follows:

1. Tissue preparation
2. De-paraffinization
3. Inactivation of non specific elements
4. Antigen retrieval
5. Blocking of endogenous peroxidase
6. Primary antibody incubation
7. Secondary antibody incubation
8. Application of counter stain (Haematoxylin)
9. Application of DAB (di-amino benzidine) substrate
10. Mounting by a suitable agent
11. Microscopic examination. [7]

The aim of this study is to compare the quality of sections stained by immunohistochemistry with two different methods of antigen retrieval: the microwave method and the autoclave method.

AIM: To know the concept of antigen retrieval in immunohistochemistry and compare the efficacy of antigen retrieval performed by microwave treatment thereupon of autoclave method.

OBJECTIVES:

1. To evaluate immunohistochemical sections subjected to antigen retrieval by microwave treatment method.
2. To assess immunohistochemical sections subjected to antigen retrieval by autoclave method.
3. To match the morphology of the immunohistochemical sections during which antigen retrieval has been done by microwave treatment method thereupon of immunohistochemical sections during which antigen retrieval (AR) has been administered by autoclave method
4. To assess which method of antigen retrieval is best fitted to obtaining top quality immunohistochemical sections in routine histopathological examination.

MATERIALS & METHODS:

The present study is of prospective and analytical type and will be conducted for duration of two years in the histopathology and immunohistochemistry divisions of department of Pathology, J.N.M.C. Due approval will be taken from the Institutional ethics panel (I.E.C) and consent will be taken from the patients participating within the study. The study will evaluate immunohistochemical sections during which antigen retrieval would be done either by microwave treatment method or autoclave method in 30 cases. All the cases are going to be divided in two groups:

- Group 1 during which antigen retrieval has been performed by microwave treatment method[8].
- Group 2 during which antigen retrieval has been administered by autoclave method.

INCLUSION CRITERIA:

All the cases where the paraffin embedded tissue blocks are available are going to be included within the study.

EXCLUSION CRITERIA:

Cases where the paraffin embedded tissue blocks aren't available are going to be excluded from the study.

APPROACH TO THE PRESENT STUDY:

- Approval from I.E.C.
- Duly informed consent (if needed).
- Selection of appropriate paraffin embedded tissue blocks with adequate tissue (either a tumor mass or normal tissue) for immunohistochemistry.
- Antigen retrieval with either microwave treatment method or pressure cooker method.
- Immunohistochemical staining by standard protocol.
- Microscopic examination of immunohistochemically stained sections.
- Comparison of morphology of the sections (AR by microwave treatment method) with those in which AR has been done by pressure cooker method.

MATERIALS REQUIRED:

- Automated tissue processor- Histokinette (Leica™ TP 1020).
- Paraffin wax.
- Induction plate for heating purpose (for preparing liquid paraffin wax for embedding).
- Leukhardt's molds (L molds).
- Rotary microtome (Leica™ RM 2125 RT).
- Egg Albumin.
- Glass slides (BLUE STAR®). Dimensions: 7.5 x 2.5 centimeters.
- Glass slide marker.
- Distilled water.
- Tissue float bath.
- Hematoxylin and Eosin stain
- Immunohistochemistry staining kit. (DAKO™, Glostrup, Denmark)
- DPX (distyrene, a plasticizer and xylene) mounting medium.
- Cover glasses (BLUE STAR®). Dimensions: 60 x 22 millimeters.

MICROWAVE TREATMENT: [9] [10]

0.01 M citrate buffer (pH: 6.0), 0.1 M Tris-Hcl buffer (pH 8.0) and 1mM EDTA sodium hydroxide solution (pH 8.0) are used as antigen retrieval solutions in this method. Sections which are re-hydrated are treated with retrieval solution and subjected to microwave treatment in an oven (750W). A total 3 cycles for 5 minutes each are done.

PRESSURE COOKER METHOD OF ANTIGEN RETRIEVAL:

Two liters of retrieval solution are placed in a stainless steel pressure cooker which has a capacity of 6 liters with an operating pressure of 103kPa which is kept to boil on a hotplate (1.5kW)

IMMUNOHISTOCHEMISTRY STAINING PROTOCOL:

PRINCIPLE: High quality nuclear, cytoplasmic and membrane staining with minimum background staining is facilitated by the Polymer-HRP detection system especially in tissues rich in endogenous biotin. Sections are treated with primary antibody which will bind the antigen. The secondary antibody is integrated along with a suitable polymer. This complex is again integrated with a suitable enzyme marker. After this, a suitable substrate is added. The substrate is acted upon by an enzyme. This process leads to a color reaction. The color of the reaction is attributed to DAB chromogen substrate.

Materials required for staining for immunohistochemistry:

- **Tri-sodium citrate buffer (for antigen retrieval):**
 - Citric acid solution, 10.51 grams in 500 milliliters of distilled water.
 - Tri-sodium citrate, 14.70 grams in 500 milliliters of distilled water.
 - Working citric acid solution (stock), 9 milliliters.
 - Working Tri-sodium citrate solution (stock), 41 milliliters.
 - Distilled water, 450 milliliters.
- **Tris buffer (for washing purpose):**
 - Tris free base, 1.5 grams.
 - Sodium chloride, 20 grams.
 - 1 N Hydrochloric acid, 10 milliliters.
 - Distilled water, 2500 milliliters.
- **1 N Hydrochloric acid (for pH adjustment):**
 - Concentrated Hydrochloric acid, 9 milliliters.
 - Distilled water, 91 milliliters.
- **4 N Sodium Hydroxide (for pH adjustment):**
 - Sodium hydroxide pellets, 16 grams.
 - Distilled water, 91 milliliters.
- Poly-L Lysine (PLL), dilution 1:10:
- PLL, 1 milliliter.
- **Distilled water, 9 milliliters.**

STAINING PROTOCOL:

- Immunohistochemistry is a modern technique in which antigens in tissues and cells are localized by antigen antibody reaction.
- An appropriate paraffin block with adequate tumor mass and satisfactory amount of normal tissue is selected.

- Sections of thickness 3 to 5 micrometers are cut and placed on PLL coated slides.
- De-paraffinization is achieved by placing the sections in xylene.
- Sections are rehydrated by subjecting them to descending concentrations of alcohol.
- Running tap water is used for washing.
- Sections are treated with distilled water for 1 minute.
- Transferring the sections to Coplin's jar containing retrieval buffer is done.
- Antigen retrieval is carried out in a pressure cooker for 15-20 minutes. The solution used is comprised of 30 ml retrieval solution in 1500 ml of distilled water.
- The pressure cooker is left to cool to the room temperature.
- Sections are dipped once in distilled water.
- Tris buffer solution is used to wash the sections for at least 5 minutes at room temperature. This step is repeated three times.
- A mixture of 3, 5 Hydrogen Peroxide and Methanol is used for peroxidase blocking which is done for 30 minutes.
- Sections are washed in Tris buffer solution 3 times for 5 minutes each.
- The antibody Brand- DAKO™ (Glostrup, Denmark) is applied at room temperature for 1 hour.
- Sections are again washed in Tris buffer solution 3 times for 5 minutes each.
- Envision technique is performed by utilizing a labelled polymer for 30 minutes 37 degree celsius.
- Sections are treated with Tris buffer solution 3 times for 5 minutes each.
- The DAB (3, 3'- diaminobenzidine) substrate is applied for 15-20 minutes. The working DAB solution is composed of 1 ml DAB buffer and 25 microliters of DAB concentrate.
- Washing of sections is done by Tris buffer, this time for 5-10 minutes.
- Sections are treated with distilled water.
- Harris's Haematoxylin is applied as counter-stain for 5 -minutes.
- Again, the sections are washed in running tap water.
- After the above process is complete, sections are dried, mounted with a suitable mounting medium and examined under the microscope.

INTERPRETATION: The antigen antibody reaction is visible as a colored complex which is localized either to the membrane, cytoplasm or the nucleus.

INTERPRETATION OF RESULTS The results will be examined by 3 observers and will be assessed as follows:

- complete negative: -
- Weakly positive in a percentage of cells expected to be positive: +---
- All cells are weakly positive which are expected to be positive: +++-
- All cells are moderately strong positive which are expected to be positive: ++++
- All cells strongly positive which are expected to be positive: ++++ [11]

STATISTICAL ANALYSIS: Will be done by SPSS software version 22.0, Prism 6.0 analysis software. Chi square test will be used for comparison.

OBSERVATIONS AND RESULTS: The data collected from observations will be analyzed and then compared with similar research. The data collected will be tabulated in a master chart and. The efficacy of antigen retrieval carried out either by pressure cooker method or microwave treatment will be compared. The quality of immunohistochemical sections will be compared and appropriate conclusions will be drawn out.

DISCUSSION:

Norton AJ et al. [8] in their study explored the advantages of microwave based antigen retrieval for diagnostic immunohistochemistry. An alternative method of antigen retrieval, heat mediated antigen retrieval was also investigated. It was observed that heating the sections in 0.01 M citrate buffer (pH 6.0) gave similar results as those obtained by microwave based antigen retrieval. Added benefits like increasing the speed of treatment, results reproducibility with large slide batches and the power to implement the use of metal slide racks were observed.

In 1991, two autonomous gatherings revealed that exposure of routine areas to high temperatures in an watery medium could improve the location of antigens by defeating the concealing effect of formal infixations. Since at that point, articles managing the problem of AR in routine segments have been flourishing in the writing, in light of warming or presentation to a strong alkali or corrosive. At present, HBAR strategies are day by day used in histopathology labs, as they have been found to be more effective and all the more effectively pertinent than other AR frameworks. Nonetheless, the fluids and warmth sources employed differ from site to site. this doesn't favour standardization in immunohistochemistry, which is felt to be significant by numerous scientists and open institutions. Even the components by which these new techniques are so effective in antigen exposing are still unclear. Cattorette and Suurmeijer suggested that 'heat and hydrolysis may both denature and break the tissue proteins at or close to the connections made by the formalin between neighboring amino acids' and thought it 'conceiv-capable that self-gathering of unfurled protein chains with subsequent reclamation of antigenic destinations happens when the retrieval arrangement is permitted to cool'. Shi et al. have stressed that the pH of the recovery arrangement is an important cofactor for certain antigens. Morgan et al. have revealed that tight complexing of calcium ions or other divalent metal cations with proteins during formaldehyde fixation can be answerable for masking certain antigens; along these lines, the chelation or precipitation of these particles can speak to a basic advance in salt-mediated AR. The extreme high temperatures may be expected to provide sufficient vitality to deliver the calcium particles and divalent metal cations from the pen like buildings that they form with proteins. A Number of related studies were reviewed [12-17]. The present investigation gives further help to the usefulness of HBAR and gives new data in order to change this 'kitchen' approach into a more standardized apparatus.[18-30]

CONCLUSION:

Appropriate conclusions will be drawn from the findings in observations and discussion.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is

absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES:

1. Jeyapradha Duraiyan, Rajeshwar Govindrajan, Murugesan Palanisamy. “Applications of Immunohistochemistry”. The Journal of Pharmacy & Bioallied Sciences. August 2012; vol 4 (2) 307-309.
2. Shi SR, key ME, Kalra KL. Antigen retrieval in formalin fixed paraffin embedded tissues, an enhancement method for Immunohistochemical staining based on microwave Owen heating of tissue sections. J Histochem Cytochem, 1991; 39: 741-48
3. Suurmeijer AJH. Microwave-stimulated antigen retrieval. A new method facilitating immunohistochemistry of formalin-fixed, paraffin embedded tissue. Histochem J 1992; 24: 597.
4. Cattoretti G, Becker HG, Key G, et al. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 1992; 168: 357–363.
5. Cattoretti G, Pileri S, Parravicini C, et al. Antigen unmasking on formalin-fixed, paraffin embedded sections. J Pathol 1993; 171: 83–98.
6. Gown AM, deWever N, Battifora H. Microwave-based antigenic un-masking. A revolutionary new technique for routine immunohistochemistry. Appl Immunohistochem 1993; 1: 256–266.
7. Suurmeijer AJH, Boon ME. Notes on the application of microwaves for antigen retrieval in paraffin and plastic sections. Eur J Morphol 1993; 31:144–150.
8. Norton AJ, Jordan S, Yeomans P. Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. J Pathol 1994; 173: 371–379.
9. Morgan JM, Navabi H, Schmid KW, Jasani B. Possible role of tissue-bound calcium ions in citrate-mediated high-temperature antigen retrieval. J Pathol,1994; 174: 301–307.
10. Flenghi L, Bigerna B, Fizzotti M, et al. Monoclonal antibodies PG-B6a and PG-B6p recognize, respectively, a highly conserved and a formol-resistant epitope on the human BCL-6 protein amino-terminal region. Am J Pathol 1996; 148: 1543–1553.
11. Pileri SA, Roncodor G, Ceccareli C, Piccioli M, Briskomatis A, Sabbatini E, Ascani S, Santini D. Antigen retrieval techniques in Immunohistochemistry: Comparison of different methods. Journal of Pathology, 1997, vol. 183: 116-123.
12. Mohite, D., A. Hande, R. Gupta, M. Chaudhary, P. Mohite, S. Patil, and M. Gawande. “Immunohistochemical Evaluation of Expression Pattern of P53, P63, and P73 in Epithelial Dysplasia.” Journal of Datta Meghe Institute of Medical Sciences University 13, no. 3 (2018): 122–29. https://doi.org/10.4103/jdmimsu.jdmimsu_64_18.
13. Nidhi, S., and G. Madhuri. “Comparative Evaluation of Immunohistochemical Expression of MT-1 MMP, TIMP-1, TGF-B1, α -SMA in Oral Submucous Fibrosis and

- Oral Submucous Fibrosis with Coexisting Oral Squamous Cell Carcinoma.” *European Journal of Molecular and Clinical Medicine* 7, no. 2 (2020): 1994–2002.
14. Rai, P.D., S. Vagha, S. Shukla, and A. Bhake. “Comparison of Various Scoring Systems by Immunohistochemistry for Evaluating Hormone Receptors (Estrogen Receptor and Progesterone Receptor) in Carcinoma of Breast.” *Journal of Datta Meghe Institute of Medical Sciences University* 15, no. 2 (2020): 202–8. https://doi.org/10.4103/jdmimsu.jdmimsu_7_20.
 15. Ranka, R., M. Chaudhary, P. Sharma, M. Gawande, A. Hande, and S. Patil. “Role of Immunohistochemical Markers in Surgical Margins of Patients with Head and Neck Carcinoma - A Systematic Review.” *International Journal of Current Research and Review* 12, no. 6 (2020): 9–17. <https://doi.org/10.31782/IJCRR.2020.12062>.
 16. James SL, Castle CD, Dingels ZV, Fox JT, Hamilton EB, Liu Z, Roberts NL, Sylte DO, Bertolacci GJ, Cunningham M, Henry NJ. Estimating global injuries morbidity and mortality: methods and data used in the Global Burden of Disease 2017 study. *Injury Prevention*. 2020 Oct 1;26(Suppl 2):i125-53.
 17. Kumar A, Chery L, Biswas C, Dubhashi N, Dutta P, Dua VK, Kacchap M, Kakati S, Khandeparkar A, Kour D, Mahajan SN. Malaria in South Asia: prevalence and control. *Acta tropica*. 2012 Mar 1;121(3):246-55.
 18. Chole RH, Patil RN, Basak A, Palandurkar K, Bhowate R. Estimation of serum malondialdehyde in oral cancer and precancer and its association with healthy individuals, gender, alcohol, and tobacco abuse. *Journal of cancer research and therapeutics*. 2010 Oct 1;6(4):487.
 19. Pradhan S, Madke B, Kabra P, Singh AL. Anti-inflammatory and immunomodulatory effects of antibiotics and their use in dermatology. *Indian journal of dermatology*. 2016 Sep;61(5):469.
 20. Acharya S, Shukla S, Mahajan SN, Diwan SK. Acute dengue myositis with rhabdomyolysis and acute renal failure. *Annals of Indian Academy of Neurology*. 2010 Jul;13(3):221.
 21. Gadbail AR, Chaudhary M, Patil S, Gawande M. Actual Proliferating Index and p53 protein expression as prognostic marker in odontogenic cysts. *Oral Diseases*. 2009 Oct;15(7):490-8.
 22. Prasad N, Bhatt M, Agarwal SK, Kohli HS, Gopalakrishnan N, Fernando E, Sahay M, Rajapurkar M, Chowdhary AR, Rathi M, Jeloka T. The adverse effect of COVID pandemic on the care of patients with kidney diseases in India. *Kidney international reports*. 2020 Sep 1;5(9):1545-50.
 23. Walia IS, Borle RM, Mehendiratta D, Yadav AO. Microbiology and antibiotic sensitivity of head and neck space infections of odontogenic origin. *Journal of maxillofacial and oral surgery*. 2014 Mar 1;13(1):16-21.
 24. Lohe VK, Degwekar SS, Bhowate RR, Kadu RP, Dangore SB. Evaluation of correlation of serum lipid profile in patients with oral cancer and precancer and its association with tobacco abuse. *Journal of oral pathology & medicine*. 2010 Feb;39(2):141-8.
 25. Korde S, Sridharan G, Gadbail A, Poornima V. Nitric oxide and oral cancer: A review. *Oral oncology*. 2012 Jun 1;48(6):475-83.
 26. Gondivkar SM, Gadbail AR. Gorham-Stout syndrome: a rare clinical entity and review of literature. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*. 2010 Feb 1;109(2):e41-8.

27. Gadbail AR, Chaudhary M, Gawande M, Hande A, Sarode S, Tekade SA, Korde S, Zade P, Bhowate R, Borle R, Patil S. Oral squamous cell carcinoma in the background of oral submucous fibrosis is a distinct clinicopathological entity with better prognosis. *Journal of Oral Pathology & Medicine*. 2017 Jul;46(6):448-53.
28. Gadre PK, Ramanojam S, Patankar A, Gadre KS. Nonvascularized bone grafting for mandibular reconstruction: myth or reality?. *Journal of Craniofacial Surgery*. 2011 Sep 1;22(5):1727-35.
29. Sorte K, Sune P, Bhake A, Shivkumar VB, Gangane N, Basak A. Quantitative assessment of DNA damage directly in lens epithelial cells from senile cataract patients. *Molecular vision*. 2011;17:1.
30. Basak S, Rajurkar MN, Mallick SK. Detection of *Blastocystis hominis*: a controversial human pathogen. *Parasitology research*. 2014 Jan;113(1):261-5.