INTRODUCTION

Estrogen is a nuclear receptor and regulator of epithelial growth, proliferation and differentiation with a key role in the development of breast cancer. College of American Pathologists has stratified the prognostic and predictive factors in breast carcinoma according to their strength and estrogen receptor has been categorised into Category-I prognostic marker which means that it has proven impact on prognosis and clinical management.

The analysis of estrogen receptor (ER) status has become the standard of care for breast carcinoma patients. It increases the likelihood of response to hormonal therapy by anti-estrogen drugs such as tamoxifen which competitively blocks the binding of estrogen receptors, thus antagonizing the transcriptional activation of genes required for tumour growth.

ABSTRACT

Objective: To compare immunohistochemical estrogen receptor expression on formalin-fixed, paraffin-embedded breast carcinoma tissue sections by using regular, extended microwave heating and pressure cooker technique for heat induced antigen retrieval.

Study Design: Quasi experimental study.

Place and Duration of Study: Department of Histopathology, Armed Forces Institute of Pathology, Rawalpindi, from August 2006 to July 2007.

Methodology: The study was conducted on 40 cases of breast carcinoma diagnosed on histopathology and selected by convenience sampling. One section each of the tumour was separately subjected to regular microwave heating (10 minutes), extended microwave heating (20 minutes) and pressure cooker (heating for 2 minutes after reaching full pressure). A nuclear staining of > 10% cells with moderate intensity was considered positive and frequency of ER expression by each technique was compared statistically. Sensitivity and specificity of the techniques was determined using pressure cooker technique as the gold standard for this study.

Results: Out of 40 cases, ER expression in 24 (60%) cases was seen by microwave regular heating (MRH) and in 30 (75%) cases by microwave extending heating (MEH) technique. Pressure cooker (PC) technique for antigen retrieval demonstrated 34 (85%) cases with ER expression. Out of 16 which were negative by MRH technique, 6 became positive by MEH while 10 became positive by PC. Statistically significant difference in ER expression by PC and MEH technique was seen in comparison to MRH with a p-value of < 0.05. Moreover, 4 cases which were negative by MEH technique turned positive for ER expression by PC. MRH and MEH had 100% specificity but sensitivity was 70.6% and 88.2% respectively taking PC technique as gold standard with diagnostic accuracy of MEH as 90% and MRH as 75%.

Conclusion: Pressure cooker antigen retrieval technique is a better method than microwave heating. The increase in duration of heating improves the percentage of positive cells as well as intensity of ER immuno-staining which entitles breast cancer patient to benefit from ER positive treatment protocols which have better prognosis.

Key words: Antigen retrieval. Estrogen receptor. Microwave. Pressure cooker heating. Ductal breast carcinoma.
has been used to unmask the antigen with special emphasis on the effects of time, temperature and pH on antigen retrieval. In a comparative study using microwave (regular and extended heating), pressure cooker, autoclave and steamer, it was concluded that different heating methods could yield similar results if the heating times were adjusted appropriately. Pressure cooker has been described as preferred method in another comparative study for heat induced antigen retrieval technique by Neves et al. Using microwave, pressure cooker, steamer and water bath.

In Pakistan, there are only limited centres offering histopathology laboratory services and even less offering immunohistochemistry evaluation. The samples are received from distant areas, with variable unstandardised fixative concentrations and prolonged fixation time ranging from 18 hours to months. Factors like nature of fixative, amount of fixative and any delay in fixation remain unknown. In the presence of above factors, it is believed that antigen retrieval would make significant difference in immunohistochemical results especially for ER expression since it has therapeutic implications. Therefore, this study was planned to compare the results of ER expression by antigen retrieval technique using microwave at regular and extended heating times with pressure cooker in cases of breast carcinoma.

METHODOLOGY

This study was carried out at Armed Forces Institute of Pathology (AFIP), Rawalpindi, from August 2006 to July 2007. AFIP is a referral laboratory with an annual histopathology workload of approximately 26,000 surgical biopsy specimens. It receives samples from armed forces hospital establishments, Northern Punjab, Khyber-Pakhtunkhwa and from adjoining civil and private hospitals of Rawalpindi and Islamabad region.

Among the lumpectomy or mastectomy specimens with or without axillary clearance from female breast carcinoma patients and received in 10% formalin study cases of ductal carcinoma were selected by convenience sampling. Representative sections of the tumour were taken and processed for paraffin embedding. Tissue sections of 3-4 µm thickness were made and stained with hematoxylin and eosin (H and E) for confirmation of histological diagnosis. Cases having received neo-adjuvant chemotherapy or a diagnosis of malignancy other than ductal carcinoma were excluded from the study.

Three sections of 3-5 µm thickness were made from each paraffin block of microscopically confirmed ductal carcinomas and placed on clean glass slides with a pre-attached adhesive on its surface for immunohistochemistry. A known ER positive case was also used as a positive control during each batch of immunohistochemistry for quality control.

Deparaffinization of the sections was carried out in xylene, followed by clearing with alcohol and rehydration in Tris Buffered Saline (TBS). Each section of the same tumour was subjected to a separate heat induced antigen retrieval technique. Slides were placed in a domestic microwave (750 watts) in a heatproof tray for two cycles of 5 minutes each with a total heating time of 10 minutes for regular microwave heating (MRH) for antigen retrieval. In extended microwave heating (MEH) technique, slides were subjected to four cycles of 5 minutes each with a total time of 20 minutes. Similarly, slides were kept in a pressure cooker in 0.1 M Tris-HCl at pH 10 for full 2 minutes after boiling and reaching full pressure.

The slides were cooled down at room temperature and washed twice with TBS for 02 minutes after subjecting to antigen retrieval using different heating techniques. For immunostaining, the slides were first treated with primary antibody (ID5 of Zymed laboratories) for one hour followed by three changes of TBS for 3 minutes each. Biotinylated secondary antibody was placed on the slides for 20 minutes followed again by three changes of TBS of 3 minutes. Enzyme conjugate was applied for 2 minutes, after which slides were given washing in TBS. Towards the end, diaminobenzidine chromogen (DAB) was applied for 10 minutes and slides were rinsed in distilled water followed by counterstain with haematoxylin and mounted with Canada balsam.

The slides were screened manually. A nuclear staining of more than 10% of tumour cells assessed at low power in combination with a moderate staining intensity estimated at high power was taken as positive for ER expression.

The data were collected and results were entered in SPSS for the three different techniques. Descriptive statistics were determined for the percentage expression of ER by three different techniques. The techniques were compared with one and other using Chi-square and Fisher's Exact test taking a p-value of < 0.05 as significant. Sensitivity and specificity of the MRH and MEH technique were determined along with positive predictive value, negative predictive value and diagnostic accuracy taking PC technique as gold standard.

RESULTS

There were 40 cases of breast carcinoma amongst which 24 (60%) cases showed positive ER expression using MRH technique for antigen retrieval. The number of positive cases with ER expression using MEH was 30 (75%) cases while PC technique showed 34 (85%) ER expression on the same cases.

Amongst the 16 cases which were negative by MRH technique, 6 became positive by MEH while 10 became positive by PC and in the end out of 40 cases only 6
cases remained negative using three different techniques for antigen retrieval. None of the 24 cases which were positive by MRH technique, become negative either on MEH or PC technique. There was significant difference in ER expression by both PC and MEH technique in comparison to MRH with p-value of < 0.05 (Table I).

On evaluating the MEH and PC technique, it was observed that 4 cases which were negative by MEH technique showed positive ER expression by PC technique (Figure 1). Similarly, all the cases which were negative for ER expression by PC technique also remained negative by MEH technique. Comparison of the two techniques showed a p-value of < 0.05 which was significant.

**Table I**: Comparison of microwave regular heating with pressure cooker and microwave extended heating technique for antigen retrieval.

<table>
<thead>
<tr>
<th>Pressure Cooker</th>
<th>Microwave Regular Heating</th>
<th>Positive cases</th>
<th>Negative cases</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive cases</td>
<td>24</td>
<td>10</td>
<td>34</td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>Negative cases</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>16</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table II**: Comparison of microwave extended heating (MEH) and microwave regular heating (MRH) technique for antigen retrieval taking pressure cooker (PC) as Gold standard (n=40).

<table>
<thead>
<tr>
<th>Pressure Cooker</th>
<th>Total</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Diagnostic accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microwave extended heating</td>
<td>Positive cases 30</td>
<td>0</td>
<td>30</td>
<td>88.2%</td>
<td>100%</td>
<td>60%</td>
</tr>
<tr>
<td>Negative cases</td>
<td>4</td>
<td>6</td>
<td>10</td>
<td>70.6%</td>
<td>100%</td>
<td>37.5%</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>6</td>
<td>40</td>
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Since PC technique had the highest expression in comparison to the other techniques, it was taken as gold standard technique for this study. Sensitivity of the MEH and MRH techniques was 88.2% and 70.6% respectively while specificity was 100% for both techniques. The negative predictive value of MEH technique was 60% with a diagnostic accuracy of 90% while negative predictive value of MRH technique was 37.5% with a diagnostic accuracy of 75% (Table II).

![Figure 1](https://example.com/figure1.png)
Table III shows the comparative summary of the negative cases by MRH technique which showed improvement in the percentage of cells as well as the staining intensity of cell for ER expression by MEH and PC technique. The MEH technique showed an increase in the percentage of cells as well as the intensity of staining in individual cases comparison to the MRH technique. Moreover, the cells which did not show any expression by MRH and MEH were positive in 20-90% of cells of the cases by PC technique. However, the staining intensity of the cells which were positive on both MEH and PC technique remained more or less the same as moderate in intensity.

DISCUSSION

Epidemiological studies confirm breast cancer as the commonest malignancy of females in Pakistan according to Karachi Tumour Registry and AFIP tumour registry. Prognosis and management of breast cancer is influenced by the classic variables including the status of hormonal receptors - estrogen receptor (ER) and progesterone receptor (PR). The basic aim of detection of ER positivity is to assess if a given patient can benefit from adjuvant endocrine therapy. Therefore, immunohistochemical analysis of ER needs careful attention to ensure adequate quality assurance. Only a few studies regarding ER expression by immunohistochemistry have been done in Pakistan. Azizun-Nisa et al. showed an expression of 32.7%. Fatima et al. showed ER expression of 40.5% in a study of 150 cases each while Haider et al. demonstrated 54% ER expression in their study of 100 cases. Estrogen expression as low as 34% has also been reported using microwave antigen retrieval method. In contrast to the above studies, Sharif et al. showed 72.3% ER expression in females and 81% in males, which is close to the present study by MEH and pressure cooker antigen retrieval technique. Indian literature reports ER expression between 51-57% in breast cancers. At the same time Dunwald et al. reported ER expression in breast carcinoma as up to 80%. In another large series of 5,993 cases of breast carcinomas as Nadji et al. showed 75% ER expression. Using microwave antigen retrieval technique Jotti et al. showed 60% ER expression in their study of 119 cases. Present study showed ER expression ranging from 60-85% ER expression on the same tumour sections using three different heat induced antigen retrieval techniques.

Variable expression of ER suggests marked inter-laboratory variation and false negative results for ER expression in breast carcinoma patients. This is a subject of concern and emphasises upon the need of quality assurance in histopathology. Antigen retrieval is the most important factor for achieving accurate and consistent results. Efficiency of the antigen-retrieval step has been highlighted as the single most important factor contributing to inter-laboratory variability for ER expression according to data obtained from 66 laboratories participating in a United Kingdom external quality assurance programme. The current comparative study also shows statistically significant difference between MRH, MEH and PC heat induced antigen retrieval techniques. Results of pressure cooker technique for antigen retrieval, which achieves a higher temperature (115°C or higher), are better than those laboratories that used microwave technology (100°C). Pressure cooker technique has been found to be more convenient on practical grounds, as it allows simultaneous handling of a large number of slides and also economises on time by as low as one minute for the heat retrieval procedure. Other studies also favour the pressure cooker as a better method of antigen retrieval. Comparative studies by Neves et al. and Norton et al. using microwave, pressure cooker, steamer and water bath revealed pressure cooker as a preferred method. The present study also showed greater, up to 85%, ER expression by pressure cooker with brisk nuclear staining and clear background in comparison with the microwave extended and regular heating methods.

In a NEQAS-ICC report, the length of time for heat antigen retrieval has been identified as the most important variable for improving ER testing standardization. Extending the antigen retrieval heating time to 25 minutes provided the most consistent results between participants irrespective of buffer, buffer pH or heating mechanism used. In present study, a maximum heating time of 20 minutes was used which showed a statistically significant improvement in results in comparison to 10 minutes heating time. There were 6 cases which were negative at regular heating and became positive on extended heating. However, intense heating for 20 minutes did result in evaporation of buffer solution and dryness of sections. It is recommended that there should be enough quantity of TBS in order to minimise loss via evaporation during heating. Another problem encountered in the study was that sections with greater adipose tissue content were washed out on extensive heating by microwave and by pressure cooker as also observed by Shousha et al. Therefore, a good adhesive is important for enduring the extensive heat and pressure cooker methods to prevent them from being washed away.

Standardization remains the main issue of recent concern. It involves the three main areas including antibodies/reagents, technical procedure and interpretation of reports. Keeping these points in view, Shi et al. concluded that it is impossible to standardize immunohistochemistry for accurate quantification in formalin.
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fixed paraffin embedded tissue sections unless an optimal reference control material is included and treated in a manner identical to the test specimen. Therefore, each laboratory should establish its standardized heat antigen retrieval time. Internal and external controls should be used routinely with each batch to ensure the accuracy of the processing technique as well as the staining reactions of the antibody and other reagents.

CONCLUSION

The presently used antigen retrieval methods enable laboratories to detect ER by IHC with greater sensitivity and specificity than was possible with previous techniques. Among the heat induced antigen retrieval techniques, pressure cooker heating is a better method than the microwave heating. The increase in duration of heating improves the percentage of positive cells as well as intensity of ER immunostaining which entitles the patient to benefit from ER positive treatment protocols which have a better prognosis.

REFERENCES