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The Effect of Washing After Protein Blocking on Immunohistochemical Staining Applied to Paraffin Sections

Parafin Kesitlere Uygulanan İmmünohistokimyasal Boyamada Protein Blokajı Sonrası Yıkama Yapılmasının Sonuca Etkisi

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ABSTRACT

Aim: Immunohistochemistry is still frequently preferred in both diagnostic and experimental studies because it can show proteins where they are in the tissue. One of the main problems in immunohistochemistry is the background staining, which can be prevented by protein blocking and which occurs as a result of binding of primary antibodies to tissue proteins and Fc receptors in the tissue due to antigenic similarity. There is no consensus on whether to wash after protein blocking in current publications and immunohistochemistry manuals published by manufacturers.

Material and Methods: In our study, routine immunohistochemistry procedure was applied to determine the expression of TNF-α on 5 μm thick sections obtained from rat gastric tissue samples in which an experimental gastric ulcer model was created with ethanol, and two groups were formed, with and without washing after protein blocking, with 10 sections in each group. For semi-quantitative evaluation, the histological score (h-score) was calculated from the images obtained from the immunohistochemically stained preparations of both groups and the obtained data were statistically compared.

Results: As a result of our study, no statistically significant difference was found between the h-score values of the groups that were washed after protein blocking in immunohistochemical staining and the groups that did not wash (p=0.971). The median (min-max) values of the groups with and without washing are 211 (179-244) and 215 (171-251), respectively.

Conclusion: In the immunohistochemical staining procedure, washing after protein blocking does not change the staining intensity, does not create background staining, and does not affect the result of the h-score for semiquantitative evaluation. Similar studies are recommended for other species, tissue types and antigens.

Keywords: Immunohistochemistry, paraffin section, protein blocking, washing, h-score

ÖZ

Amaç: İmmünohistokimya, proteinleri dokuda bulundukları yerde gösterebilmesi nedeniyle gerek tanısal gerek ise deneysel çalışmalarda günümüzde halen sıklıkla tercih edilmektedir. İmmünohistokimyada temel problemlerden biri de, protein bloklama yapılarak önlenebilen ve antijenik benzerlik nedeniyle doku proteinlerine ve dokudaki Fc reseptörlerine primer antikorların bağlanması sonucu ortaya çıkan

zemin boyanmasıdır. Güncel yayınlarda ve üreticiler tarafından yayımlanan immünohistokimya el kitaplarında protein bloklama sonrası yıkama yapılıp yapılmaması konusunda fikir birliği yoktur.

Gereç ve Yöntemler: Çalışmamızda etanol ile deneysel mide ülseri modeli oluşturulmuş sıçan mide doku örneklerinden elde edilen 5 *µ*m kalınlığındaki kesitlere TNF-α ifadesini belirlemek amacıyla rutin immünohistokimya prosedürü uygulanmış ve her grupta kesit sayısı 10 olacak şekilde protein bloklama sonrası yıkama yapılan ve yapılmayan iki grup oluşturulmuştur. Her iki grubun immünohistokimyasal yöntemle boyanmış preparatlarından elde edilen görseller üzerinden semikantitatif değerlendirme amacıyla histolojik skor (h-skoru) çıkarılmış ve elde edilen veriler istatistiksel olarak karşılaştırılmıştır.

Bulgular: Çalışmamızın sonucunda immünohistokimyasal boyamada protein bloklama sonrası yıkama yapılan grup ile yıkama yapılmayan grupların h-skor değerleri arasında istatistiksel olarak anlamlı fark bulunmamıştır (p=0,971). Yıkama yapılan ve yapılmayan gruplara ait medyan (min-maks) değerleri sırasıyla 211 (179-244) ve 215 (171-251)'dir.

Sonuç: İmmünohistokimyasal boyama prosedüründe protein bloklama sonrası yıkama yapılması zemin boyanması oluşturmamakta ve semikantitatif değerlendirme için yapılan h-skoru sonucunu etkilememektedir. Diğer doku tipleri ve antijenler için de benzer çalışmalar yapılması önerilmektedir.

Anahtar Sözcükler: İmmünohistokimya, parafin kesit, protein bloklama, yıkama, h-skor

INTRODUCTION

Immunohistochemistry (IHC) is a labeling method that enables to show antigenic structures in their localization in tissue or cell by using antibodies specific to the antigen to be detected. The ability to display the antigen in situ is the most important advantage of IHC, and this advantage has led to its widespread use in both diagnostic and research laboratories for decades (1). Although the demand for the use of IHC has increased since the mid-1980s, studies on the standardization and optimization of IHC protocols have been limited (2).

One of the most important problems researchers face when evaluating the IHC result is background staining. Over the years, strategies such as enzyme blocking, biotin blocking and protein blocking have been developed to overcome background staining by considering parameters such as the tissue type and IHC method used (3). Although background staining due to endogenous enzyme and biotin has been overcome with reagents produced by manufacturers, non-specific antigen-antibody interactions still exist as an important cause of background staining (4).

The main cause of background staining due to antigen-antibody interaction is thought to be the interaction of Fc fragments of primary and secondary antibodies with Fc receptors in the tissue, and it poses an important problem for the investigator to face, especially in the case of polyclonal antibody use (5). Treatment of tissue with normal serum from the same species as the secondary antibody or with a commercially produced universal blocking agent prior to incubation of the tissue with the primary antibody can prevent non-specific antigen-antibody interactions (6). However, there is a difference of opinion on whether to wash the tissue with phosphate buffered saline (PBS) after incubation with these reagents. Several research articles, reviews, and IHC manuals published by manufacturers state that no wash should be performed after protein blocking, regardless of tissue and antigen type, while others state that washing is vital to remove excess protein that may prevent detection of the target antigen (1,7-9).

The aim of our study is to give a new perspective to the mystery about whether washing should be done after protein blockage in the method of IHC, which is widely used in both diagnostic and research laboratories today.

MATERIAL and METHODS

In our study, paraffin-embedded gastric tissue obtained from a rat, in which an experimental gastric ulcer model was created with ethanol in another study, was used. For this purpose, the ethics committee approval numbered 2023-02-02/02 was obtained from the Animal Experiments Local Ethics Committee of Zonguldak Bülent Ecevit University.

Consecutive sections of 5*µ*m thick were taken from the paraffin block using Shandon Finesse 325 brand cylindrical microtome. With the sections obtained, two groups were formed that were washed with PBS after protein blockade and not washed (sections 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and sections 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, respectively) (n=10). The number of sections in the groups was determined using similar previous studies (10,11). Our study was carried out within the framework of an evidence-based medicine program. For this reason, 3rd term students of Zonguldak Bülent Ecevit University Faculty of Medicine were included in the study. Sections from the paraffin block and immunohistochemical staining process were carried out together with the students. Sections in both groups were stained by the indirect immunohistochemical method (ABC method) as described below.

Deparaffinized sections were placed in citrate buffer (pH 6), antigen retrieval was applied in a microwave oven to expose the antigenic binding sites, and left to cool at room temperature. After washing with PBS, sections treated with Triton X for permeabilization were washed again with PBS and

outlined with a hydrophobic pen. To neutralize endogenous peroxidase activity, 3% H_2O_2 was applied for 20 minutes. Sections were treated with Ultra V block (Thermo Fisher, Massachusetts, USA) for seven minutes in order to mask the non-specific binding sites, then one group was washed with PBS while the other group was not washed. Washing was performed as previously described by slowly dipping and removing the tissues into PBS 2-3 times (8,9).Then, sections of both groups were incubated with anti-TNF-α (polyclonal, 1:200 dilution, Sigma Aldrich, Germany) primary antibody for 24 hours at +4 °C. Sections washed with PBS were treated with biotin-conjugated secondary antibody (Thermo Fisher, Massachusetts, USA) and streptoavidin-peroxidase complex (Thermo Fisher, Massachusetts, USA) for 30 and 10 minutes, respectively. Sections treated with diaminobenzidine (DAB) chromogen for 45 seconds under a light microscope were counterstained with hematoxylin, covered with entellan, and examined under a Zeiss Axio Lab A1 light microscope.

For the semiquantitative evaluation of the immunohistochemical staining result, histological scoring (h-score) was performed using the following criteria; 0; no staining, 1+; weak staining, 2+; moderate or prominent staining, 3+; intense coloring. 10 fields in each section were scored according to the above criteria at x40 objective magnification under a light microscope, and the arithmetic mean of the values obtained from 10 areas was accepted as the h-score of that section. The h-score value for each area was obtained by multiplying the percentage of stained cells for each density category by its density. Average scores obtained were used for statistical analysis. h-score = ∑i i xPi, i; density score, Pi; cell percentage (12). The h score obtained from the immunohistochemical staining images was evaluated by two histologists-blinded manner.

Statistical Analyses

Statistical evaluations were made using the Jamovi 2.3.21 program. Descriptive statistics were expressed as median (minimum-maximum). The Mann-Whitney U test was used to compare the two groups, and $p<0.05$ was considered significant for all evaluations.

RESULTS

The sections of the groups that were washed and not washed after blocking were evaluated double-blindly by two histologist, and the h score was calculated. The median (min-max) values of the washing and non-washing groups are 211 (179-244) and 215 (171-251), respectively. There was no significant difference between the groups in the statistical analysis of the h-score data calculated for the semiquantitative evaluation of IHC staining result (p=0.971) (Figure 1, Table 1).

In both groups, it was observed that cells with different staining intensities in the cytoplasm and cells with no staining coexisted in accordance with the cellular localization of TNF-α. When the IHC staining results of both groups were evaluated qualitatively in terms of background staining and specific staining, it was observed that there was no significant observational difference between the groups in terms of staining properties. Although it was observed that the number of cells with (+)1 staining intensity in some areas was higher in the no washing group than in the washing group, this did not affect the h-score result (Figure 2).

DISCUSSION

Background staining is the most challenging problem for researchers in the IHC technique during the evaluation of the results. There are numerous reasons for background staining, such as incorrect fixative selection, inappropriate primary antibody concentration, length of chromogen application time, and failure to neutralize endogenous biotin and enzyme activities (13). One of the reasons for background staining is the binding of primary and secondary antibodies to Fc receptors in the tissue (4). To overcome this problem, non-immune serum or universal blocking agents are used (14). However, after this stage, two different views emerged about washing the tissue with PBS (15,16). In our study, the effect of washing after protein blockade on the results of immunohistochemical staining was examined.

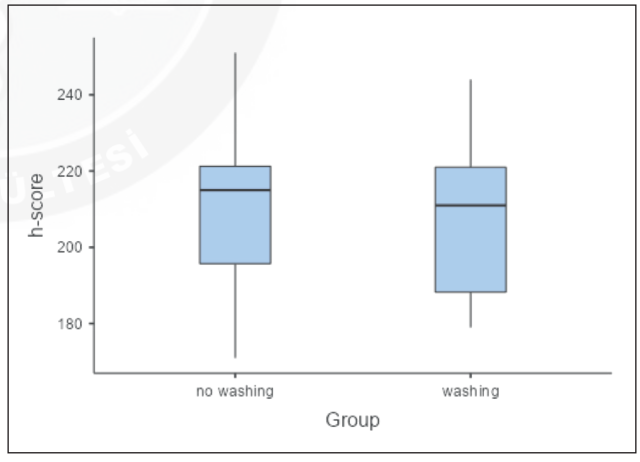

Figure 1: h-score results of groups. Values are given as median (min-max). (p=0.971)

Table 1: H-score values for TNF-α expression of groups. Values are given as median (min-max).

Protein	Washing group (n=10)	No washing group (n=10)	p value
TNF-α	211 (179-244)	215 (171-251)	0.971

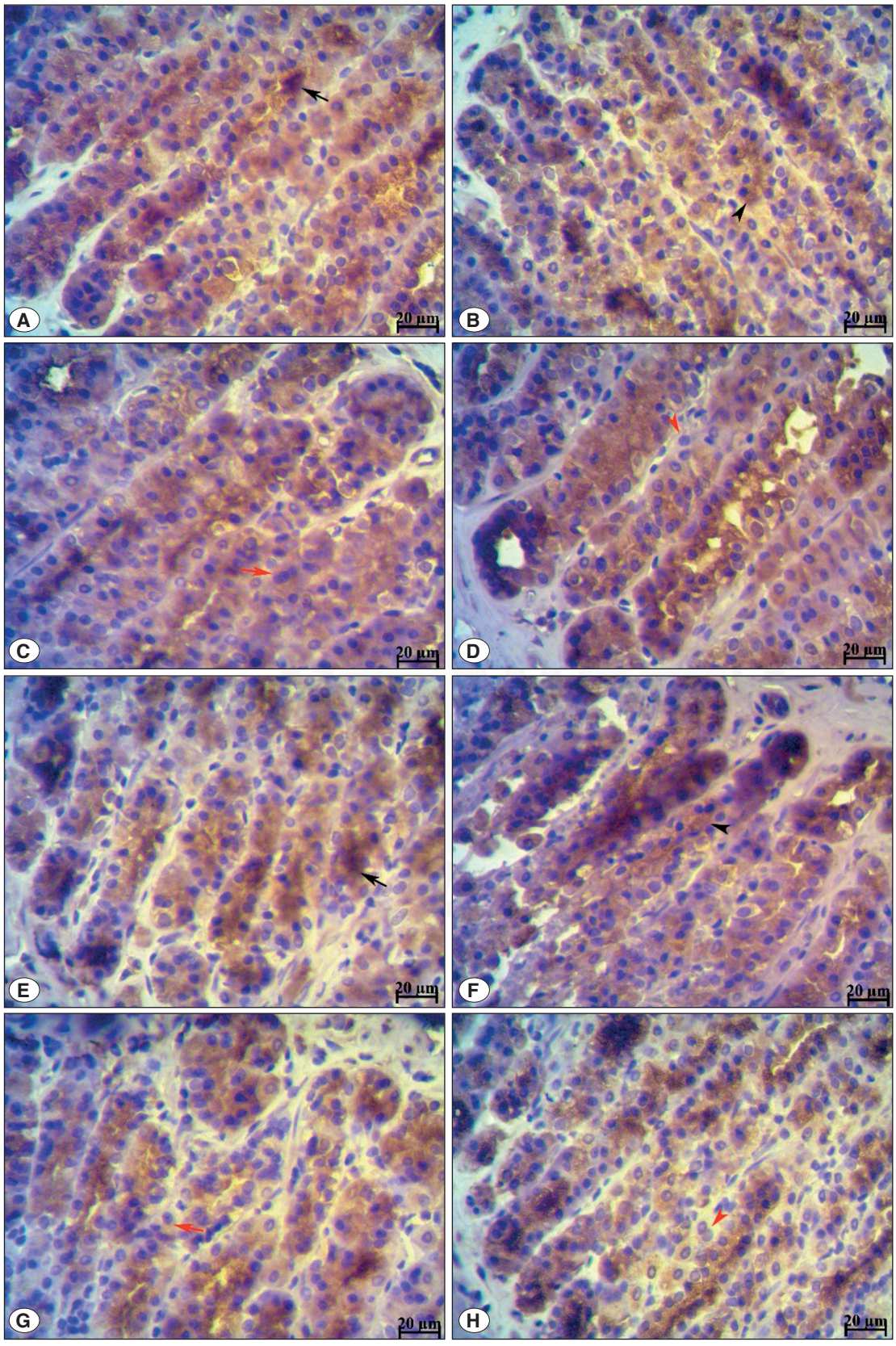

Figure 2: Immunohistochemical staining images of the groups. **A-D)** no washing group, **E-H)** washing group. Black arrow; 3 positive staining, black arrowhead; 2 positive staining, red arrow; 1 positive staining, red arrowhead; no staining Scale bar; 20 μm.

We strictly followed the manufacturer's directives in terms of the standardization of the IHC protocol we apply. In this study, in which we compared the h-score values obtained from the two groups, we could not find a significant difference between the two groups.

Buchwalow et al, in a study they conducted with different human tissues and antibody types, showed that the absence of protein blockade did not affect the immunohistochemical staining result, and this may be due to the loss of the ability of Fc receptors in the tissue to bind to the Fc fragment of IgG after formaldehyde fixation (4).

However, it is still accepted that antigen detection in immunohistochemistry is possible through specific binding between antibody and epitope, that this binding is governed by hydrophobic interactions, ionic interactions, hydrogen bonding and other intermolecular forces, respectively, and that the same attractive forces/bonds may also contribute to nonspecific binding (17).

In the light of this information, it was concluded that washing did not affect both the chemical bonds involved in the antigen-antibody interaction and the background staining caused by the interaction of the Fc part of the primary antibodies with the Fc receptors in the tissue. It is thought that further studies with different species, tissues and antibodies will contribute to the explanation of the mechanism.

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Author Contributions

Mete Keçeci designed the study. **Mete Keçeci, Elif Koca, Esra Arslan, Reyyan Başak Denek, Ezgi Su Sarı, İpek Pınar Akpınar, Bünyamin Ülker, Kağan Atıcı, Özgenur Erbakan** performed the sectioning and immunohistochemical staining process. **Mete Keçeci, Osman Cengil** made the h-score. **Mete Keçeci, Elif Koca, Esra Arslan, Reyyan Başak Denek, Ezgi Su Sarı, İpek Pınar Akpınar, Bünyamin Ülker, Kağan Atıcı, Özgenur Erbakan** contributed to the literature review and article writing processes.

Conflicts of Interest

The authors have no relevant financial or non-financial interests to disclose.

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None.

Ethical Approval

Ethics committee approval numbered 2023-02-02/02 was obtained from Zonguldak Bülent Ecevit University Animal Experiments Local Ethics Committee for the study.

Review Process

Extremely peer-reviewed.

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