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Computer Assisted Image Analysis of Peroxidase Stained Endometrial Tissue

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Aim: In immunohistochemistry, antibodies are used to detect and localize antigens in cellular compartments in sections taken from frozen or paraffin blocks. Currently, computerized image processing techniques are widely available to analyze immunohistochemically stained tissues; however, the image analysis programs are generally highly specific and expensive. We aimed to show the feasibility of computer assisted image analysis (CAIA) by using two computer programs currently in widespread use.

Methods: Two available computer programs, Adobe Photoshop CS and ImageJ, were used to separate areas in the same tissue sections stained with NOS antibody and labeled with peroxidase in human endometrial tissue. CAIA was made on the relevant images using these two programs.

Results: There was a significant difference among the groups according to staining intensity, in all three grades of staining ($P < 0.001$, $P < 0.05$, and $P < 0.001$, respectively). Subjective assessment of the images was in good agreement with objective CAIA. One (+) positive stained area was greater in group 1 and less in group 2 and group 3 ($P < 0.001$ and $P < 0.001$, respectively). Group 3, which was considered as the most intensely stained by the observer, had the greatest amount of ++ staining.

Conclusion: CAIA is a safe and reliable method of analyzing tissue samples stained using immunohistochemical methods.

Key Words: Computer, image analysis, immunohistochemistry, scoring

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Peroksidaz İle Boyanmış İnsan Endometrium Dokusunun Bilgisayar Destekli Görüntü Analizi

Amaç: İmmünohistokimyasal incelemede parafinde bloklanmış veya donmuş kesitlerde, hücre yapılarındaki antijenleri saptamak ve yerlerini belirlemek için antikorlar kullanılır. Günümüzde immünohistokimyasal olarak boyanmış dokuları incelemek için bilgisayar destekli görüntü analiz teknikleri bir hayli yaygındır, ancak bu amaçla kullanılan programlar oldukça pahalıdır. Bu çalışmada, yaygın olarak bulunabilen iki bilgisayar programı kullanarak bilgisayar destekli görüntü analizi (BDGA) yapmanın mümkün olduğunu göstermeyi hedefledik.

Yöntemler: İnsan endometrial dokusunda NOS antikorunu ile boyanmış ve peroksidaz ile işaretlenmiş alanları ayırt etmek için yaygın olarak bulunan Adobe Photoshop CS ve ImageJ bilgisayar programları kullanıldı. İlgili doku resimlerinde bu iki program kullanılarak BDGA teknikleri uygulandı.

Bulgular: Boyanma yoğunluğuna göre resim grupları arasında her bir boyanma şiddeti düzeyinde anlamlı bir fark vardı ($P < 0.001$, $P < 0.05$ ve $P < 0.001$). Resimlerin öznel değerlendirilmesi, nesnel olarak yapılan BDGA ile uyum içerisindeydi. Bir (+) pozitif boyanan alanlar 1. grupta en fazla, grup 2 ve 3'de ise daha azdı ($P < 0.001$ ve $P < 0.001$). Gözlemci tarafından en yoğun boyanmış olarak değerlendirilen grup 3 ise ++ boyanmanın en fazla saptandığı gruptu.

Sonuç: BDGA, immünohistokimyasal teknikler kullanılarak boyanan dokuları incelemek için güvenilir ve etkin bir yoldur.

Anahtar Sözcükler: Bilgisayar, görüntü işleme, immünohistokimya, değerlendirme

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Introduction

Since its inception in the 1940's, immunohistochemistry has gradually developed into a major diagnostic tool in cellular pathology. The identification of specific or highly selective cellular epitopes, which are usually protein in nature, in routinely processed frozen or paraffin embedded tissues with an antibody and appropriate labeling system, has had a significant impact on histological diagnosis, especially over the last 15 years (1). In immunohistochemistry, antibodies are used to detect and localize antigens in cellular compartments in sections taken from frozen or paraffin blocks. An antigen can

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be easily identified using immunohistochemistry; however, one can not answer the given questions by knowing only of the presence or absence of the antigen in the tissue. Antigen detection depends on the specificity of the primary antibody and localization on the ability to resolve different compartments. In a great number of experiments, precise description of differences in staining patterns or intensity among sets of tissue samples is required. This kind of description could be made by quantification of the antigen staining in the tissue. The staining of antibodies can be evaluated semiquantitatively using HSCORE method, and is calculated using the following equation: $HSCORE = \sum P_i (i+1)$, where i = intensity of staining with a value of 1, 2 or 3 (weak, moderate, or strong, respectively) and P_i is the percentage of stained cells at each intensity, varying from 0% to 100% (2).

Manual quantification is labor-intensive and requires multiple observers to adhere to rigid parameters to maintain inter-observer agreement, and it provides only semi-quantifiable data (3). Image processing techniques devoted to the study of biological specimens have made many valuable contributions to science by providing new and highly accurate information about the objects being studied. The introduction of computers and the increase in computational power have enabled researchers to analyze the images by the aid of a computer. Computer assisted image analysis (CAIA) is a method used to separate tissue elements from the background. The separated elements can be subjected to further image analysis (4). There are quite a number of image analysis programs; however, they are usually an additional burden to a research project budget. The ImageJ is an image analysis program developed at the National Institutes of Health (5). It is in the public domain and freeware, and can be further extended with the use of plug-ins developed for this program.

In this study, we aimed to analyze staining intensity in NOS-stained images of endometrial tissue, using a CAIA method in two widely available programs, Adobe Photoshop CS and ImageJ. We evaluated the images and used CAIA methods in order to highlight the areas stained by peroxidase.

Materials and Methods

Endometrial samples were obtained from the Department of Obstetrics and Gynecology at the

University of Celal Bayar, Manisa, Turkey. The samples were taken from healthy fertile women (n=15) who were admitted to our hospital for surgical sterilization, during the post-ovulatory 7th or 8th day of their menstrual cycle, i.e. in the implantation period.

Endometrial samples obtained using a sampling device (Pipelle de Cornier, Paris, France) were evaluated and dated according to the Noyes criteria for confirming the timing of the sampling procedure (6). The Research Ethics Committee of the Medical School of Celal Bayar University approved our study protocol, and all subjects provided informed consent.

Immunohistochemistry

Endometrial biopsies were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for a maximum of 24 h. They were embedded in paraffin following a routine embedding procedure. Cross-sections of 5 μ were stained with primary antibodies: anti-iNOS in a 1:1000 dilution (Zymed, South San Francisco, CA, USA) for 18 h. After washing, the secondary antibody (biotinylated goat IgG anti-rabbit/mouse IgG) was applied for 30 min, followed by three washes in PBS. The streptavidin-peroxidase complex was added for 30 min and washed with PBS three times. Slides were counter-stained with Mayer's hematoxylin, dehydrated, and cleared, and after the application of a cover-slip, they were analyzed on a light microscope with BX 40 (Olympus, Tokyo, Japan). The presence of brown precipitate indicated positive findings for the primary antibody. The negative staining controls received the same treatment, with rabbit IgG or mouse IgG instead of the primary antibody. Serial sections were examined and staining patterns were compared. Two observers who were blinded to the clinical information of the endometrial samples independently evaluated the staining scores.

Computer Assisted Image Analysis

Images were digitally acquired using the Coolpix 5400 camera (Nikon, Japan). The images were grouped by an experienced observer according to staining intensity. The groups consisted of three grades of staining intensity: there were 7 images in the + positive stained group (group 1), 4 images in the ++ stained group (group 2),

and 4 images in the +++ positive group (group 3), respectively.

Images were stored in "jpg" format and transported to a PC. The images were opened using the Photoshop CS program. A sample image was chosen from the image set and a representative histochemically stained area was selected with the rectangular selection tool. This particular area was chosen as it contained different degrees of staining (Figure 1). The colors in the + positive stained area were selected by the color range command of the select menu (Select>Color Range). The "Add to Sample" color selector tool of the "Color Range" dialog box was used to select different shades of + stained areas. The degree of staining intensity was graded manually. The fuzziness value was set as 30. The selected range of colors was then saved as an Adobe color range file format with an extension of "axt"; this file was named "positive1.axt". At the original image, the areas containing + positive staining were selected and the pixels in these areas were deleted. This step was done in order to not accidentally select the same color values for the ++ and +++ positive areas. Two additional color range files

were created for the ++ and +++ positive areas using the same methodology. Color range files were created by another observer who was unaware of the grouping of the image.

The action palette was opened in the Adobe Photoshop and the following actions were recorded as a script. A new set of actions for + positive staining was created, and named as positive 1 action. The Select>Color Range command was invoked and the color range file for + positive staining was chosen by the load command of the color range dialog box (Figure 2). The hard edges of the selection were softened by a degree of 2 pixels by the Select>Feather command. The selection was then copied, a new layer was then created named as positive 1 layer, and the selection was pasted to this new layer. The pixels with + positive stained areas were on a new layer which was named as positive 1. The same actions were recorded for the ++ and +++ positive stained areas using the related color range files. In the end, each image had three different layers, each one representing a different staining intensity. These independent layers were then saved as independent "jpg" files.

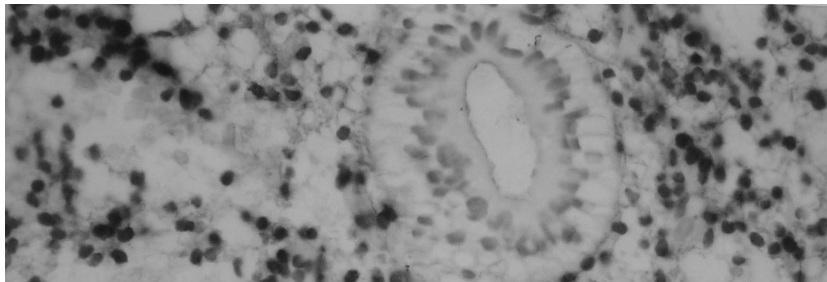


Figure 1. The endometrium tissue stained with anti-iNOS antibody using indirect immunoperoxidase technique, the stained parts are in a brownish color. (x400 original magnification).

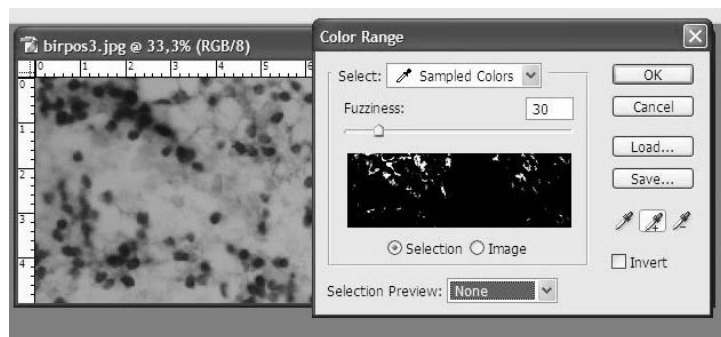


Figure 2. The dialog box of color select command.

The images were cropped to a size of 425x762 pixels with a resolution of 300 dpi; the stained area was included in the cropped image (Figure 3). Great care was taken to ensure the same amount of stained tissue was included in these images; all further image analysis was done on these images. The resultant image was opened using ImageJ, and the image was thresholded using the Process>Binary>Threshold command. This command converts grayscale images to black and white and color images to eight primary colors. In order to smooth the thresholded image, Process>Filters>Mean filter was used, which works by replacing each pixel with the neighborhood mean. Three pixels were set to be the size of the neighborhood. In order to reduce noise in the image, Process>Filters>Maximum filter was used. This filter does grayscale erosion by replacing each pixel in the image with the smallest pixel value in that pixel's neighborhood. The image was thresholded again (Figure 4), and the size and number of particles in the images were measured using the "Minimum Size" option of the "Analyze Particles" dialog box. The analyze particles command gave the number of the particles and the area of each particle. These results were exported to SPSS 11.0 and statistical analysis was done.

Statistical Analysis

The stained areas were compared among the three groups. The criterion for grouping was the subjective assessment of the observer as explained in the Materials and Methods section. Kruskal-Wallis test was used as a nonparametric ANOVA test, and Dunn test as a post-hoc test where appropriate.

Results

The +, ++, and +++ positive stained areas, as measured using CAIA methods, were significantly different among the three different study groups. The + positive staining area was significantly different among the study groups ($p < 0.001$). The + positive staining area was greater in group 1 and decreased in groups 2 and 3, respectively (Figure 5) ($p < 0.05$ and $p < 0.001$, respectively). The ++ positive staining area was also significantly different among study groups ($p < 0.0001$). The ++ positive area was significantly greater in group 3 than group 2. The +++ positive staining area was greater in groups 2 and 3 than group 1 ($p < 0.001$ and $p < 0.001$, respectively). There was no significant difference between



Figure 3. The + positive selected areas as selected by the + color range file.



Figure 4. The thresholded image showing the stained areas; as processed by ImageJ program.

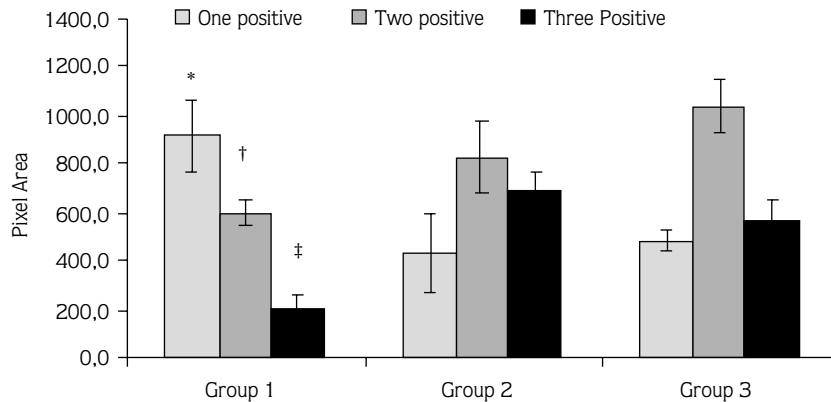


Figure 5. The graph showing the pixel area of three different grades of staining among study groups: There was a statistically significant difference among the study groups according to pixel area for all of the three different grades of staining intensity ($p < 0.001$, $p = 0.04$, and $p = 0.0004$ respectively).

*; group 1 > group 2 ($p < 0.01$), group 1 > group 3 ($p < 0.001$), group 2 > group 3 ($p < 0.05$).

†; group 2 < group 3 ($p < 0.05$).

‡; group 1 < group 2, group 1 < group 3 ($p < 0.05$).

groups 2 and 3 according to the +++ positive stained areas.

Discussion

The results obtained by CAIA were in good agreement with the subjective judgment of the observer. One plus positive stained area was greater in group 1, which was graded as + positive stained by the observer. Likewise, + positive staining was decreased in groups 2 and 3. Two plus staining was most intense in group 3 - this result seems like a deviation from the subjective judgment of the observer, as the images in group 3 was deemed as having the most intense staining by the observer. However, it is apparent from CAIA that the reason for the observer to assign the images in group 3 was the abundant presence of ++ positive stained areas in these images. The intense staining in group 3 was actually caused by the increased area of ++ positive staining, which could not be judged by the naked eye. This result could be regarded as indicating a precision of CAIA.

There are two main approaches to delineate areas of interest using CAIA: one can either select positively the area of interest or one can use a color subtraction (CS) method where the background is selected and deleted until only the areas of the interest remain in the image

(7,8). Underwood et al. (9) used a CS-CAIA method to delineate nerves in the subcutaneous tissue of diabetic rats. Positive descriptions of color must be broad enough to include all the features of interest and strict enough to exclude background. This scenario often creates a color description that is frequently specific to each image, exhibiting impaired accommodation of variations in color hues among different images. Therefore, we used a CS method to create the color range files. First, we selected mildly stained, namely + positive areas, and we then deleted them from the image in order to not select the same color values again. Using this approach, we were able to separate areas stained with peroxidase from the rest of the image.

CAIA is not free from human intervention. In our methodology, the color range files are generated manually by an observer, and this could be a source of error. However, when proper color range files are created and the same color range files are used in a group of images, qualitative data from a large sample can be obtained in a very short time. The process can give highly detailed data about a particular area of interest very quickly. On the other hand, the standardized method of HSCORE calculation is heavily dependent on human observation and is labor-intensive. While HSCORE is a standard method in which the percentage of the stained

cells and the intensity of staining are taken into account, CAIA is not (2). Some modification of our method, which takes into account the number of cell nuclei in the image, must be done in order to have a CAIA equivalent of HSCORE. This issue could be a subject of further investigations.

The selection of images for CAIA is very important - the human eye can detect artifactual areas; however, CAIA can not separate true staining from artifactual staining. The images to be processed by CAIA must be free from artifacts. The area of tissue in all of the images to be analyzed must be nearly the same. CAIA can erroneously generate a lower score for a heavily stained tissue if the amount of stained tissue in the image is small. In order to standardize our measurements, we cropped images to the same size and included the same amount of tissue in all images.

There are a certain number of programs which perform CAIA; however, as they are expensive, the

majority of investigators in Turkey do not have access to them. We are aware that the Adobe Photoshop CS program is a commercial product; however, the Gimp program is a free clone of Photoshop which can be obtained at the web address <http://www.gimp.org/>. It has a similar but more basic function, but is an open source and plug-ins can be written to upgrade this program. CS-CAIA methods can be used with the Gimp program as it can only select one color at a time. This will possibly require extensive work on a single image. The functionality of the Adobe Photoshop program can be rendered to the Gimp program when a similar function is implemented. The power of the Adobe Photoshop program is that it can generate color range files for selection. It is possible to write such plug-ins for ImageJ, if a color range selection feature can be obtained. This will enable the investigators on a limited budget to evaluate histochemical staining in tissue samples.

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