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Immunohistochemestry vs. Immunofluorescence: Comparative analysis via software of total colorimetric reaction of GPR43 protein in adipose tissue

Silvia Gisell Vega-Damián[1], Eugenio Torres-García[1], Rigoberto Oros-Pantoja[1]*, Jorge Luis De-la-Rosa-Arana[2], Daniela Rodríguez-Muñoz[3], Elia Yadira Peniche-Moreno[4]

[1] Faculty of Medicine, Autonomous University of the State of Mexico, Zip. 50180, Toluca, Mexico; E-Mail: sgvdamian@hotmail.com

[2] Immunoparasitology Lab. Institute of Epidemiological Diagnostic and Reference; Ministry of Health Zip.01480, Mexico City.

[3] Morphology and citology laboratory. Postgraduate studies and research section. Biological Sciences National School. National Polytechnic Institute. Zip. 11340. Mexico City.

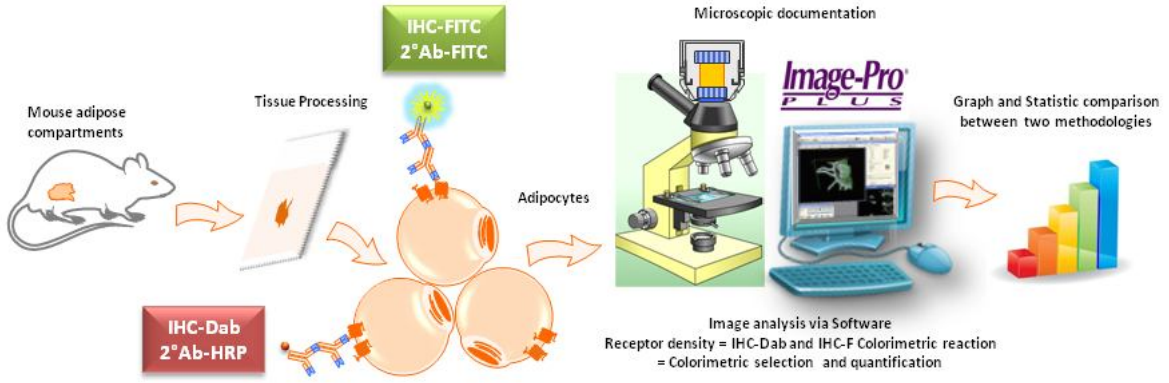
[4] Pathologic Anatomy Lab. Children's Hospital. Mexico's State Maternal and Child Institute. Zip. 50170, Toluca, Mexico.

***** Rigoberto Oros-Pantoja; Faculty of Medicine, Autonomous University of the State of Mexico; Neuroscience Departament. Paseo Tollocan esq. Jesus Carranza S/N, Toluca, Mexico. Zip. 50180. E-Mail: rigoberto_ros@hotmail.com
Tel.: +52 (722) 217 3552; 217 3577; (ext. 218).

Abstract: Immunohistochemistry revealed by diaminobenzidine (IHC-DAB) or fluorescence (IHC-F) are two of the most common techniques used in histopathology. In order to evaluate the similarity between these methodologies that share the same antigen-antibody reaction principle, using Image-Pro Plus software we performed total colorimetry quantification and comparison of immunohistochemical reaction; revealed with diaminobenzidine (IHC-DAB) and FITC fluorescence (IHC-F). As biological model, GPR43 protein was assessed by immunohistochemistry in inguinal, mesenteric and gonadal (WAT) cd1 mice adipose tissue. In predefined areas of digital images, we evaluated the reaction obtained following the same processing conditions. Results: The GPR43 protein in both IHC-DAB and IHC-F was evidenced in adipocytes membrane and infiltrated immune cells. The average of positive reaction in μ^2 (total colorimetry x μ^2) on a predefined area or region of interest (ROI) of 25x104 μm^2, showed no significant differences in the distinct tissues between the performed methods. This colorimetric similarity between the two techniques, besides supporting the quantitative analysis of

IHC-DAB, indicates that the latter could be comparable to the IHC-F, with the advantage of being used in retrospective studies. Conclusions: In the total colorimetric analysis between the two methodologies IHC-DAB and IHC-F, the resulting quantitative values were similar in all three compartments of adipose tissue studied. Subsequent studies could validate the IHC-DAB respect to IHC-F.

Keywords: *Immunohistochemistry, immunofluorescence, colorimetric-analysis, GPR43, adipocytes.*



Graphical Abstract : The figure shows the methodological sequence to compare colorimetric reaction between two indirect immunoreactions (IHC-DAB and IHC-F) via Software

Introduction: Pathology is a multidisciplinary science that besides studying structural and functional disease abnormalities from development to its senescent stage[1], it is based on morphofunctional areas of comparative biology and medicine. Its clinical components are: epidemiological, etiologic and physiopathogenic factors[2]. Contemporary pathology continues using microscopy for describing abnormal processes using a wide range of dyes and chromogens to demonstrate biological responses[3]. Pathognomonic disease findings were subsequently supported by the incursion of molecular pathology, where the use of biomarkers (antibodies) is and will remain as an essential tool for diagnosis[4]. In addition to morphological aspects evidenced by dyes, immunohistochemistry revealed by diaminobenzidine (IHC-DAB) or fluorescence (IHC-F) are two of the most common methodologies used in histopathology[5,6]. In IHC-DAB various chromogens determine the enzymatic reaction on conjugated antibodies (Avidin-Biotin Complex Peroxidase ABC or alternate labeling method) and the resulting color (e.g. brown color intensity) indicates the presence of the antigen of interest. Although several studies quantify IHC-DAB from digital images, it still prevails as a semiquantitative technique, where the score assigned depends on the observer appreciation. In immunofluorescence, when a specific wavelength excites the antibody coupled fluorochrome, physical changes occur in the

molecule. The electronic excitation results in fluorescence emission. The antigen-antibody reactions can have variable distribution therefore fluorescence emitted corresponds to a particular antigen density. In this context, the mean fluorescence intensity is conditioned by the sample quality, magnitude-time of exposure to light source, the filters quality and the maximum fluorescence emitted. However in a scanned image, you can see the total, maximum, minimum, mean and average fluorescence.

Nowadays, the integration of technological developments on optical instruments, continue to support biomedical research but also allow us to consolidate new pathology disciplines, such as computational pathology and telepathology[7,8]. In abnormality patterns on histopathologic image, new software tools allow accurate and automated analysis that may be applied in "omics" studies, databases and mathematical prediction. It is noteworthy referring that the appreciation limitations, interpretation and analysis of a digital image are subjected to variables, such as; the reagents quality and quantity, tissue fixation, tissue section thickness, dye/antibodies/chromogen incubation time. For immunofluorescence also counts the time between fluorochrome incubation and the sample exposure to natural/laser/halogen light sources. For histopathology using digital image, there is a growing number of software e.g. Aperio, Lucia, MetaView, Metamorph, ImageJ, Scion, Adobe Photoshop, Image Pro Plus[9]. In turn, these are provided with multiple applications to optimize the analysis and automated processes (macros, filters, extracting regions of interest (ROI), colocalization, interposing images, measuring and cell count).

On the other hand, GPR43 protein is a molecule expressed on the intestinal epithelium surface, lymphocytes, muscle, platelets and adipose tissue. It is a short-chain fatty acids receptor produced by intestinal microbiota; involved in energy metabolism, adipose tissue differentiation and obesity development[10]. Given the growing demand in obesity studies, the GPR43 protein is considered as a potential therapeutic target[11]. In this context a few qualitative reports do exist on GPR43protein detection via IHC-DAB or IHC-F[12,13]. For the IHC reactions colorimetric quantification, adipose tissue was selected due to the adipocytes large size and less cell interposition. For the aim of this study, inguinal, mesenteric and gonadal adipose tissue samples (WAT) were processed with IHC-DAB and IHC-F to evaluate the GPR43 protein density on adipocytes. Comparative analysis of the total colorimetry IHC-DAB and IHC-F on the documented images was performed.

Materials and Methods: The procedures were performed in cd1 healthy male mice (24 weeks-age, 35-40g. n=6), in compliance with the Mexican NOM-062-ZOO-1999 and international bioethical standards. Mice were euthanized by CO_2 chamber. Visceral, inguinal and gonadal adipose tissue were dissected, fixated in 10% buffered formaldehyde and embedded in paraffin; 4µm sections were obtained and

indirect immunohistochemistry was performed using anti-FFAR2/GPR43 (Santa Cruz-LS-A1578, dilution 1:50) primary antibody, anti-rabbit HRP IgG (Biolegend-406401, dilution 1:100) secondary antibody and DAB staining. The same primary antibody was used for immunofluorescence, replacing the peroxidated secondary antibody with anti-donkey FITC IgG (Biolegend-406403, 1:100 dilution). In both, indirect IHC-DAB and IHC- F, the primary antibody incubation lasted 12h, and 1.5h for the secondary antibodies. Fresh IHC-DAB slides were evaluated in Axiostar Carl-Zeiss microscope. IHC-F (FITC) reactions were analyzed with a microscope coupled Epi-IV FI fluorescence condenser, filter-487710 (blue excitation) with 50W halogen source. Digital photos (Tiff) were taken with the same white balance by IScapture® software; the images were captured at 400X magnification with Cooled-CCD Tucsen camera (5MP). In a predefined area or region of interest (ROI 25x104 µm2), the colorimetric reactions IHC-DAB and IHC-F were manually selected, using Image-Pro Plus 5.1 software (Media-Cybernetics, Silver Spring, MD). Subsequently a range of luminance was delimit (85-255 units) in the red histogram (RGB format), it was recorded as template and applied in subsequent images. In IHC-F, the total fluorescence emitted was quantified. The colorimetric reactions were compared with Man-Whitney U test.

Results and Discussion: Regarding quantitative analysis, there was no significant difference comparing the GPR43 protein colorimetric results for aforementioned methodologies (IHC-DAB vs. IHC-F). GPR43 immunoreactivity was documented in both adipocyte membrane and in immune cells infiltrates which also express this protein (Figure 1). Total colorimetry expressed in μm^2 and quantified on a ROI of $25x10^4 \mu m^2$, in inguinal tissue was 2429.1 ± 985.3 (IHC-DAB) vs. 2362 ± 882.7 (IHC-F) in visceral tissue 2532.8 ± 361.6 vs. 371 ± 2614.1 and in gonadal tissue 3737.7 ± 309.8 vs. 4013.6 ± 554.8. Therefore a similarity was observed between the two methods (Figure 2). The equivalence between both methods indicates similar antigen-antibody reactions. Hence IHC-DAB further than support quantification and comparison with IHC-F may be used in retrospective studies.

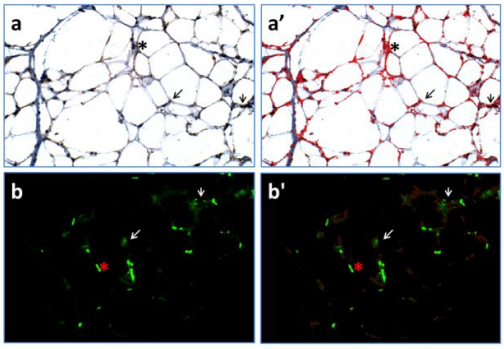


Figure 1. Colorimetric comparison of gonadal adipose tissue photomicrographs by Immnohistochemistry. a) Reaction revealed by DAB and; a') Corresponding colorimetric selection in red (dark arrows); b) Immunohistochemistry reaction by immunofluorescence (FITC), and; b') Colorimetric selection of immunofluorescence reaction outlined in red (light arrows). Star (*); representation of lymphocytic infiltrate. Paraffin sections of 4µm, 400x magnification.

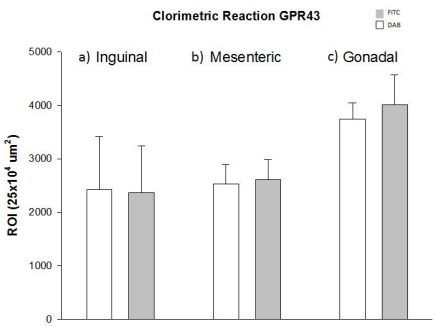


Figure 2. Statistical comparison of data obtained by immunohistochemistry. a) Comparison between DAB and FITC in inguinal adipose tissue b) Comparison between DAB and FITC in mesenteric adipose tissue c) Comparison between DAB and FITC in gonadal adipose tissue. Mann Whitney U, confidence level of 95%

Nowadays, histopathological software tools keep improving[9]; however it remains a lack of terms that unifies the criteria for quantitative analysis between different software and the comparison of IHC-DAB with other methodologies. Moreover few studies in individual methodologies perform a colorimetric quantification of an IHC reaction [14] and in most of them, only a score is assigned to distribution and quantification of cell immunoreactiviy.

Some studies used these tools in order to automate, assist and increase efficiency in pathology diagnosis and research purposes. For example in a pathologist-assisted scoring based study, using software tools (Metamorph, Aperio, Definiens Image Analysis), both methodologies were applied to different samples; the apoptotic index was quantified in IHC-F and the positive reaction expressed in pixels within a predefined area was quantified in IHC-DAB[15]. In the present study, the previous focal calibration of microscopic lenses within Image-Pro Plus software, allows the conversion of pixels into μm^2. Studies that compare quantitatively IHC-DAB vs. IHC-F on the same tissue were not yet found.

Conclusions: The equivalence between both methods indicates similar antigen-antibody reactions. Therefore IHC-DAB further than support quantification and comparison with IHC-F may be used in retrospective studies.

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Conflicts of Interest: The authors declare no conflict of interest

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