

Protocols of confocal microscopy to study vascular dysfunction in Diabetes mellitus

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Diabetes is characterized by high blood glucose levels due to autoimmune destruction of islet β -cells, or insufficient insulin secretion, or glucose non-responsive to production of insulin by β -cells. Diabetic vasculopathies, important occurrences of both type 1 and type 2 diabetes are related to the major complications of the disease, such as nephropathy, neuropathies, and metabolic disorders. The establishment of the disease and its damages can be evidenced through protocols designed for confocal microscopy. The increasing development of confocal images acquisition methods has supported these protocols to enhance knowledge ranging from macro environment to molecular mechanisms. Studies concerning the diagnosis and treatment of diabetes are highly benefited by technical procedures that involve such specific protocols. By using specific fluorescent probes (fluorophores), it is possible to investigate the installation and the progression of the vascular disorder in studies done *in vivo*, *ex vivo*, and *in vitro*. In this sense it is necessary to design accurate protocols in order to optimize the use of the available set of probes and fluorescent indicators. This chapter aims to provide a mini-review addressing the use of confocal microscopy techniques to support the comprehension of vascular disorder installation in diabetes as well as of the progression and diagnosis of the disease. Protocols and sample preparations developed in our laboratory and their findings will be described in parallel with the literature data.

Keywords: diabetic vascular disorders; fluorescence confocal microscopy in diabetes; confocal techniques in diabetic vasculopathy

1. Introduction

The production of insulin by β -cells [1] is involved in Diabetes Mellitus, which is characterized by a vascular dysfunction that results from disturbances in the endothelial release of vasoactive factors affecting muscular signaling pathways underlying vasorelaxation and vasoconstriction. In turn, this vascular dysfunction underlies the development of diabetic macro and microvascular complications, such as retinopathy, nephropathy, cardiopathy and vasculopathy. [2]. Methods of image acquisition are important tools to study molecular mechanisms [3-6], including in the ones in diabetic conditions.

Although Restini and Bendhack [7] have reviewed the applications of confocal microscopy in the study of vascular biology in general, vascular damages in the diabetes present peculiar conditions that require some specific approaches, for example, to get the better probe performance.

One of the major aspects from the endothelial dysfunction triggered by Diabetes is the impairment of production and bioavailability of the relaxant factor nitric oxide (NO) [8]. The mechanisms underlying endothelial and muscular dysfunction evoked by Diabetes mostly result from oxidative stress, which in turn results from increased bioavailability of reactive oxygen species (ROS) due to both enhanced activity of pro-oxidant enzymes induced by hyperglycaemia and to the reduced ROS inactivation by antioxidant systems, also affected by high glucose [5, 8-10]. For instance, superoxide anion (O_2^-) impairs NO generation by leading to NOS uncoupling. Furthermore, O_2^- also reduces NO bioavailability by reacting with NO thus leading to peroxynitrite ($ONOO^-$) formation [8]. Then, one of the most important experimental approaches in studying diabetic vascular dysfunction lies on assessing vascular oxidative stress and NO levels by confocal microscopy. This chapter intends to briefly describe the technical features and the main limitations of these protocols while focusing on living vascular tissues from diabetic animals.

2. Evaluation of diabetic vascular oxidative stress through measurement of O_2^- intracellular levels by confocal microscopy

The main ROS that can be found in the intracellular compartment of vascular tissues of diabetic animals are O_2^- , hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and $ONOO^-$ [5, 8-13]. In general, O_2^- underlies the vascular generation of the other ROS in diabetic conditions, since superoxide-dismutase (SOD)-induced or spontaneous dismutation of O_2^- forms H_2O_2 , which can be converted into $\cdot OH$ by the Fenton reaction [9,10, 14], whereas the generation of $ONOO^-$ depends on the reaction of O_2^- with NO [8]. Thus, the first approach in evaluating vascular oxidative stress triggered by Diabetes is the measurement of O_2^- levels.

In cellular systems, it is not possible to accurately estimate the intracellular levels of O_2^- , because it is readily scavenged by SOD, NO, glutathione, vitamin C and E, or quickly self-dismutated, which shortens its half-life [15].

With this in mind, some probes were developed to promptly react with O_2^- and generate stable products that can be measured to estimate O_2^- levels [16-20]. Currently, it is possible to find didydroethidine (DHE, also known as hydroethidine, HE), lucigenin and cyclic nitron spin traps [15]. Among these probes, DHE is considered the “gold standart” to detect and measure O_2^- intracellular levels [20]; lucigenin’s redox-cycling (that leads to self-generation of O_2^-) [21-23] and nitron’s slow reaction with O_2^- (that leads to the generation of unstable O_2^- adduct) [24-26] limits their use.

Dihydroethidium is a non-selective, fluorescent, cell-permeable probe used to detect intracellular ROS [15, 27]. When cells are incubated with DHE, a blue fluorescence is emitted, which has been correlated to the unreacted probe. On the other hand, upon the oxidation of DHE by intracellular ROS, cells emit a red fluorescence from their nucleus [28, 29]. The red fluorescence derived from the oxidation of DHE results from the generation of different cationic products upon the probe’s reaction with O_2^- , H_2O_2 , $ONOO^-$ or Fenton’s reagent; these products then bind to nuclear DNA and emit fluorescence [29-35]. The main product formed from the reaction of DHE with O_2^- is 2-hydroxyethidium (2-OH- E^+), whose fluorescence is measured at an excitation of 480nm and at an emission of 567nm. However, the oxidation of DHE by O_2^- also generates intermediate products that react with H_2O_2 or $\cdot OH$, leading to the generation of ethidium (E^+) in a minor extent, whose fluorescence is measured at an excitation of 500-530nm and at an emission of 590-620nm [15, 27, 36]. In turn, H_2O_2 forms E^+ as the main product from the oxidation of DHE [37] in cellular systems; low-molecular weight hemoproteins (such as cytochromes) catalyzes this reaction [15]. Fenton’s reagent ($\cdot OH$ formed from H_2O_2 in the presence of iron or copper) also reacts with DHE, leading to the generation of both E^+ and 2-OH- E^+ [15]. On the other hand, $ONOO^-$ oxidizes DHE in the presence of carbon dioxide (CO_2), leading to the generation of a fluorescent product that absorbs light at an excitation of 430nm [38].

Both E^+ and 2-OH- E^+ bind to nuclear DNA and promote an increase of 40 times in the basal fluorescence of DHE [15, 39]. However, they produce an overlapping fluorescence that cannot be distinguished through confocal microscopy [15, 27, 36], which requires the use of a selective O_2^- scavenger as a positive control in these protocols in order to detect and quantify the intracellular levels of O_2^- [9]. Among the scavengers, the most used in vascular assays are polyethylenoglycol-SOD (PEG-SOD), the SOD-mimetic agent tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl), and the spin-trap tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate) [40,41]. Although the use of O_2^- scavengers is effective, SOD or SOD-mimetic agents can affect the oxidation of DHE due to the generation of H_2O_2 from the dismutation of O_2^- . Indeed, tempol increases the fluorescence emitted by DHE-loaded samples [42], providing a false-negative result from the O_2^- scavenging. This artifact tends to be more evident when the expression and/or the activity of endogenous catalase is impaired, like in vascular tissues from diabetic animals [43], which increases the accumulation of H_2O_2 in the cells and favors DHE oxidation. Thus, the best approach in detecting or quantifying O_2^- in vascular tissues from diabetic animals by DHE confocal microscopy assays is through the use of spin-traps with none or slight SOD-mimetic activity, such as tiron [9]. The only consideration that must be taken into account when using tiron as an O_2^- scavenger in DHE confocal microscopy assays is the concentration of the spin-trap; it should range between 0.1-1mmol/L, since the lowest effective concentration of tiron in scavenging O_2^- is 0.1mmol/L [9], whereas at 1mmol/L, tiron starts chelating Ca^{2+} [44], which affects biological responses mediated by this cation, including ROS generation.

3. Evaluation of diabetic endothelial dysfunction through measurement of NO levels by confocal microscopy

The endothelial NO level is the major marker of endothelial function, since NO mediates several mechanisms to keep endothelial integrity, such as vasorelaxation and inhibition of leukocyte adhesion and migration, platelet adhesion and aggregation, smooth muscle cell proliferation and apoptosis [45]. Therefore, endothelial dysfunction may be considered as a reduction in endothelial NO bioavailability.

In diabetic conditions, the increased vascular levels of O_2^- reduces endothelial NO levels due to NOS uncoupling and $ONOO^-$ formation [8]. Thus, one of the first approaches in studying diabetic endothelial dysfunction is by measuring endothelial NO levels. In confocal microscopy assays, the most common cell-permeable probe used to detect NO is 4,5-diaminofluorescein-2-diacetate (DAF-2DA). After entering the cell, DAF-2DA is hydrolyzed by intracellular esterases to 4,5-diaminofluorescein (DAF-2), a cell-impermeable product that yields the fluorescent derivate DAF-2 triazole (DAF-2T) upon nitrosation induced by NO or the product of the reaction between molecular oxygen (O_2) and NO, called dinitrogen trioxide (N_2O_3) [46, 47]. In turn, DAF-2T emits green fluorescence upon excitation at 490-495nm [48].

Despite the relative high selectivity of DAF-2DA in detecting NO [47], its use in diabetic conditions is limited due to the interference promoted by diabetic oxidative stress on the probe chemical behavior. $ONOO^-$ and H_2O_2 oxidize DAF-2 into an intermediate that reacts with NO, which leads to the escape of the reaction between NO and O_2 . Thereafter, the amount of the recovered DAF-2T is increased when compared to the NO-autoxidation-mediated nitrosation. It is likely that ROS induce the one-electron oxidation of DAF-2, leading to the generation of a free radical that triggers the formation of a nitrosamine upon the reaction with NO. From the proton transfer, the diazohydroxide arise, which in turn

forms triazolofluorescein after reacting with the vicinal amine [46]. Thus, in tissue and cell samples that NO and oxidants co-exist, such as in diabetic conditions, the intracellular oxidation of DAF-2 may increase the fluorescence produced upon NO-mediated nitrosation, giving erroneous results pointing to increase NO levels.

4. Confocal microscopy for diagnosis in diabetes

Besides preclinical experimental procedures that apply confocal microscopy, in order to understand its pathophysiology, studies toward the diagnosis and treatment of diabetes are highly benefited by technical procedures that involve specific protocols [49]. The precise detection and quantification of human diabetic peripheral neuropathy are important to define at-risk patients, anticipate deterioration and assess new therapies [50, 51]. Among the morbidities triggered by the progression of diabetes that can be diagnosed through confocal principles is the corneal neuropathy [51]. Corneal nerve fiber damage correlates with intraepidermal nerve fiber loss and with the severity of neuropathy in diabetic patients [52]; the more prominent the pain the greater the nerve fiber loss [52]. Its quantification may be in fact a replacement marker of diabetic neuropathy.

Known approaches able to evaluate neurological deficits depend of small nerve fibers. In this sense electrophysiology and quantitative sensory to test functional alterations are applied to detect neuropathy [6]. Nevertheless, those techniques are not suitable enough to reach small fibers, for even though the earliest damage appears to be to the small fibers, the tests primarily assess large fiber dysfunction and have a limited ability to demonstrate regeneration and repair [6]. The only techniques that allow a direct examination of unmyelinated nerve fiber damage and repair are sural nerve biopsy with electron microscopy and skin-punch biopsy [53]. Both, however, are invasive procedures that require lengthy laboratory work, considerable expertise [54] and an electronic microscope, all of which cannot be advocated for routine use [55]. The presence of nerve conduction abnormality and at least a sign or symptom of neuropathy confirms diabetic sensorimotor polyneuropathy. If nerve conduction is normal, a validated measurement of small fiber neuropathy (SFN) (with class one evidence) may be used. Corneal confocal microscopy (CCM) is a noninvasive technique that can detect small sensory corneal nerve fiber loss in diabetic neuropathy [52], idiopathic small fiber neuropathy, and in others like the corneal opacity characteristic of the Fabry disease [56]. CCM provides *in-vivo* imaging of corneal nerve fibers, which is used as tool for measuring the small fiber neuropathy especially in corneal nerve fiber damage [6].

4.1 Corneal confocal microscopy to Quantify Small Nerve Fibers in Diabetic neuropathy

The confocal imaging systems currently in use are the following: the HRT Rostock Cornea Module (HRT-RCM) [57], the tandem scanning confocal microscope (TSCM, a spinning disk confocal) [58, 59], and the Confoscan 4 (a scanning slit system) [60]. According to Tavakoli and Malik [57], HRT III- RCM is the one used for the diagnostic procedure in diabetic neuropathy; the quantification of small nerve fibers involves these five basic steps: camera preparation, patient preparation, camera alignment, patient examination, and analysis.

The HRT-RCM (Heidelberg Engineering, GmbH, Dossenheim, Germany) is a laser microscope [61] that scans a 670 nm laser beam in a raster pattern over the area of interest. Its general use of a higher numerical aperture 63x objective lens (0.9 NA) produces images with outstanding resolution and contrast. In addition, its axial resolution is better than other confocal systems' (7.6 μm) [62], and its ability to make on-line 2-D composite images considerably enlarges the field of view [63]. Automated z-scans of 60 μm , produced by the internal lens drive, have been used to produce 3D-reconstructions of the anterior cornea [64, 65]. Altering the focus over larger distances, however, must be performed by manual rotation, which can cause interference with the examination and data acquisition process. Although remote control focusing has been described, a system that allows quantitative high-resolution 3-D imaging of the full-thickness cornea has yet to be reported [62, 66]. To address these limitations, Petroll et al. [67] modified the HRT-RCM hardware and software. When testing the feasibility of performing quantitative full-thickness corneal imaging *in vivo*, the enhanced system significantly expanded the potential quantitative research applications of the HRT-RCM microscope.

Petran et al, [68, 69] developed the TSCM by using a modified Nipkow disk containing optically conjugate (source/detector) pinholes arranged in Archimedean spirals. Their work led to the design of a TSCM suited for ophthalmological use [70, 71, 72]. The system uses lense movement inside the casing to vary focal plane position relative to the tip. As a consequence, the focus can be calibrated within the cornea to allow quantitative 3-D imaging [73, 74]. To collect the data, confocal microscopy through-focusing (CMTF) was developed for the TSCM [74, 75]. CMTF constantly acquires images by scanning the cornea in an epithelium-endothelium direction at constant velocity. When the images are digitized, the user can identify images of interest and record their exact z-axis depth [74, 75]. If intensity peaks corresponding to interfaces between layers are used, then accurate and reproducible measurements of corneal, epithelial and stromal thickness can be obtained [74]. This system can also evaluate depth-dependent changes in cell morphology, density, and reflectivity [76-78]. Unfortunately, the TSCM is no longer in the market.

The Confoscan 4 (Nidek Technologies Srl, Padova, Italy) is a variable-slit real-time scanning microscope with two independently adjustable slits positioned in conjugate optical planes. Rapid oscillation of a two-sided mirror scans the slit image over the plane of the cornea to produce optical sectioning in real time [79, 80]. In addition, this user-friendly

instrument incorporates automated alignment and scanning software, and the scanning slit design improves lighting thus providing images with better contrast and SNR than the TSCM. This, however, is achieved at the expense of axial resolution, measured at approximately 24 μm (as compared to 9 μm for the TSCM) [72, 81].

5. Conclusion

Vascular dysfunction underlies the development of diabetic macro and microvascular complications, such as retinopathy, nephropathy, cardiopathy and vasculopathy. The comprehension of the development of vascular disturbances in Diabetic conditions is highly enhanced by confocal microscopy. The pathophysiology of vascular disease in this condition involves abnormalities in endothelial cells, stress through reactive oxygen species, among others. In this sense the present work has shown specific protocols and dyes to obtain the best results.

The DAF probe is the indicated if considering the decreased bioavailability of NO. If considering the increased oxidative stress, however, then DHE probe is more appropriate. For both, the experimental approaches must be carefully designed, because the probes' sensitivities are threatened by viability loss due to diabetic vascular conditions. This review has presented important solutions in how to deal with the probes when studying vascular disturbances in diabetes.

Another important use of the confocal microscopy is in the diagnosis. Neuropathies observed in diabetes patients are known to be heterogeneous in terms of symptoms, neurologic involvement pattern, course, risk covariates, pathologic alterations, and underlying mechanisms. Nevertheless, it is widely accepted that vascular damage underlies them as a cause or as a consequence.

Thus, when applying confocal microscopy to the diagnosis of some diabetic comorbidity, the vascular damages are being investigated as well. Nowadays, confocal microscopy is a useful diagnostic method toward corneal fiber damage. It is possible to use the HRT Rostock Cornea Module, or the tandem scanning confocal microscope, or the Confoscan 4. The choice must consider the patient and the equipment.

Altogether, the confocal microscopy towards the study or diagnosis of Diabetes needs optimization of protocol design.

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