Chapter 5

Cell Biology of Virus Infection. The Role of Cytoskeletal Dynamics Integrity in the Effectiveness of Dengue Virus Infection

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http://dx.doi.org/10.5772/61704

Abstract

The cell biology of viral infections is the focus of this research, in which the role of the cytoskeleton in dengue virus (DENV) replication in cell cultures was evaluated by means of Nocodazole and Cytochalasin D treatments before and after of DENV infection. The potential contribution of cytoskeleton elements with/without the treatment of depolymerizing agents was evidenced and quantified by the subcellular distribution of viral proteins, virions produced, and viral protein quantification. The cytoskeleton is involved in DENV replication because treatments with actin microfilaments and microtubule depolymerizing agents in non-cytotoxic concentrations, affected DENV2 replication in Vero cells and decreased both the viral protein expression and infectious virion production, when compared with non-treated cells. The actin and microtubules are partly involved in DENV2 replication, since the treatment does not completely blocked viral replication, suggesting that these components are necessary but not sufficient alone for DENV2 replication in Vero cells. The structural and functional role of actin and the microtubules in replication are postulated here, opening new perspectives for understanding the architecture of the replicative complex and viral morphogenesis processes, due to the role of the cytoskeleton in the organization, recruitment, and function of the cellular elements necessary for the assembly of viral factories.

Keywords: Cell biology, infection, dengue virus, cytoskeleton, viral replication

1. Introduction

The cell biology of viral infection, as a new scientific approach, considers the cell as the structural and functional unit of viral infection. Within this new research program there are four topics:

1. Virus and the cytoskeleton.
2. Virus and endomembrane systems.
3. Virus and signal cascades.
4. Virus and the nucleus–nuclear envelope [1].

There are a lot of publications already covering these four points of the cell biology of virus infection; however, here we will focus only on the role of the cytoskeleton on DENV infection. Although many years ago these studies could be classified as "virus–host cell interactions" as it was usually considered in many virology journals, since the onset of the new millennium and the advent of converged methodologies/fields of research it is possible to take another view of viral infections. Recent developments in confocal microscopy hardware, image capture and collection with augmented resolution using charge-coupled device (CCD) cameras, digital image processing, improvements in the genetic engineering of green fluorescent protein (GFP) and the chemistry of fluorophores, together with the increased capacity of computational tools for taking thousands of images, have created innovative conditions permitting the understanding of viral infections as a whole [2, 3]. Several researchers consider viruses as tools, or molecular scalpels, which are very useful for dissecting complex molecular and cellular mechanisms [4]. This chapter will consider the cycle of Virus Dengue Serotype 2 (DENV2) as a series of steps in which the virus takes control of the cytoskeleton from host cells [5].

The possibility of real-time visualization of viral infections was reached by means of genetic manipulation of the cells and viruses – cells with fluorescent subcellular compartments and viruses with fluorescent tags [6, 7]; this will be not considered here since the main goal of our approach is to study complete virions within host cells. In this context, molecular studies of isolated viral proteins in a cellular environment are not considered under the cell biology of viral infections discipline, however, those approaches are important if they are following previous research in cell biology of viral infections, because these reductionist studies can offer details regarding the identification of molecular actors in the complex cellular environment of viral infection.

On the other hand, the study of intracellular invaders [5], considering not only viruses but also bacteria, fungi, and protozoan parasites, which aims to find a cellular platform for microbiology research with the eukaryotic host cell as the main actor, is defined as cellular microbiology [8]. Since viruses are obligated parasites, they entirely depend on the cellular machinery to carry out its replication cycle; in this sense for enveloped viruses the intracellular–vesicle trafficking and the cytoskeleton play a decisive role, mainly because both of them offer structural and functional platforms where many RNA viruses are assembled and whose morphogenesis occurs in the cytoplasm of known viral factories [9, 10]. From this viewpoint, DENV will be considered as one experimental system for studying the cell biology of viral infections – its complex interactions could throw light on the search for disease therapies.

http://dx.doi.org/10.5772/61704

Dengue is emerging globally as the most important arboviral disease threatening human populations. It is caused by the *Flavivirus* DENV [11, 12], which is a small and enveloped virus with a single-stranded, positive-sense RNA, which can be translated into a single polyprotein by a host cell and viral proteins [1, 13], and is transmitted to humans by *Aedes* genus mosquitoes [14-17]. There are nearly 2.5 billion people at risk of infection with DENV in tropical and subtropical countries. It is endemic in more than 100 countries and about 100 million cases of dengue fever are estimated annually, with over 500,000 cases being the potentially fatal dengue hemorrhagic fever [12]. Dengue virus is therefore associated with high socio-economic impacts [18, 19].

The availability of vaccines and drugs for RNA viruses has become a much greater challenge than expected, because RNA viruses evolution/mutation rates continue to elude both, vaccine design and drug effectiveness, generating extensive drug-resistant mutants, and therefore over time creating far more virulent strains [20-23]. Therefore, searching for answers inside the cell may offer an alternative method to combat the virus. Recently Villar and collaborators [24], in a big clinical trial implicating several Latin American countries, have shown important protection percentages (greater than 60%), representing an important advance in the fight against the first viral infection in Colombia. In spite of these encouraging clinical responses in patients given the tetravalent vaccine for dengue, it is necessary to wait for several years in order to evaluate the impact of this vaccine has in changing the epidemiological data of the disease. Since such vaccines take time to develop or be assessed, hope could come in the form of the development of antiviral drugs directed to cellular/molecular targets in host cells.

In this way, the cell biology of viral infections [1] has matured considerably in recent years, with cytoskeleton–virus interactions being of particular interest, because it plays both a structural and functional roles in several steps of the viral replication cycle [25, 26], connecting the cell with its environment and participating decisively in the spreading of the virus to neighboring uninfected cells [27]. The virus cannot be transported into the cell by diffusion like small molecules can, therefore, the cytoskeleton and other intracellular structures become a barrier to these [28]. This is why many viruses have shown that viral infection can cause extensive and sophisticated cytoskeletal rearrangements [10, 29-31] and alterations in the endomembrane system, something required for the replication and assembly of new virions [10, 32]. All of this leads to a cytopathic effect, which is widely known and distinctive for viral infection in cellular cultures [32]. This structural and functional intimate interaction between intracellular pathogens and the cell, involves the three elements of the cytoskeleton (actin, microtubules, and intermediate filaments) [30, 33, 34].The involvement of the cytoskeleton in viral infection has been studied for over 30 years [35], and has been found to be involved throughout the life cycle of different viruses. There are many descriptions of its involvement for adenovirus [36], human and equine herpes virus [37], HIV [38], HTLV-1 [27], Rous Sarcoma Virus [39], poliovirus [40], Epstein–Barr Virus [41], human respiratory syncytial virus [42], SV40 [43], Vaccinia virus [44, 45], poliedrosis [46], papiloma [47], and pseudorabies [48]. The replication cycle of DENV has been extensively studied with particular regard to early events such as binding, fusion, uncoating, and intracellular transport of viral proteins [28, 49, 50]. On the other hand, the relationships between the replication of DENV and the cytoskeleton host

cell are not fully characterized, although data in some literature, concerning influenza and other viruses, support their existence [13, 51-56].

A structural and functional integration of vesicle trafficking and cytoskeletal/endomembrane systems in viral replication and morphogenesis represents the viral factories redefinition [10, 57, 58]. It has been postulated that trafficking occurs in microtubule motor proteins (entry via dynein and exit through kinesin [59]), something which explains the translocation of proteins between the two routes in the microtubule organizing center (MTOC) [59]. Specifically, with respect to DENV, cytoskeleton and endo/exocytosis have not been studied comprehensively [60], especially where there is dependence between vimentin intermediate filaments and the DENV infection, in which microtubules now were apparently necessary. Intercalating fluorophores were used to label the envelopes of virions associated with cholesterol isolated DENV [50, 61]; tracking was achieved in the early events of cell entry, but this did not shed new light on the endomembrane system and cytoskeleton. It has also been reported that DENV entry by endocytosis, which is pH dependent and mediated by clathrin [62], and the involvement of actin microfilaments and microtubules in mosquito cells DENV had infected was important because the inhibition of these cytoskeleton components decreased infection by 80%. The small Rho GTPases are a protein family which among other cellular functions, govern cytoskeleton reorganization [63]. When isoprenylation of Rho GTPases was blocked, using statins, the incidence of HIV[64] and DENV [65, 66] infection was reduced via alteration of the virus assembly pathway in the rough endoplasmic reticulum.

The interactions between specific viral components and different molecular complexes of the host cell are fundamental in determining the infection rate. By such reasoning, cell biological characterization of the host–pathogen interactions, and knowledge of the possible role of the basic components of the cytoskeleton, microtubules, and actin microfilaments, allow the elucidation of some mechanisms involved in the pathogenesis of DENV. This allows the identification of new potential host targets that could be used to design efficient and rational antiviral therapies.

The applied focus of the cell biology of viral infections is to searching for antiviral therapies based in cellular targets, which have special importance, because the mutation rates of cellular genes are several thousands of times smaller than the antivirals directed to the specific viral proteins. To illustrate this there are several reports of antiviral candidates blocking cellular targets in the host (Src kinase), like the works of Yang and collaborators [67, 68], and some reports from our working group where the HMG-CoA was blocked by lovastatin on assays *in vitro* and *in vivo* [65, 69]. Additionally, we have found out that ubiquitin–proteasome and cytoskeletal elements seem altered after dengue infected cells were treated with curcumin [70]. However, the most relevant finding in this approach is that one protein (IFITM, interferon-induced transmembrane protein) could be altered for blocking different viruses belonging to several families [71].

In order to determine the contribution of the two cytoskeleton elements (actin and microtubules) in DENV replication, a series of experimental assays was completed using Cytochalasin

D and Nocodazole concomitantly using a high preservation/fixation method on the cytoskeleton in order to analyze cellular images taken using fluorescent microscopy in combination with viral titration and quantification of viral proteins. This gave a better understanding of the participation of the cytoskeleton on the functional and structural issues of DENV replication. The data shown here are in agreement with previous reports from several authors confirming distinct roles in the viral infections of DENV [72-76]. The high quality of the subcellular resolution of cytoskeleton elements in this research actually reinforces their role in these structures in DENV replication.

2. Results and discussion

2.1. Preservation/ fixation of the cytoskeleton in infected cells

To understand the cytoskeletons involvement in the DENV replication cycle and virus–host cell interaction, it is necessary to make appropriate preservations with minimal levels of perturbations to the cell morphology. To test which components of the cytoskeleton (microtubules and actin microfilaments) play a role in DENV infection, a method for preserving and fixing the cytoskeleton should be implemented [39, 40], because the conventional preparations for fluorescence microscopy with methanol/acetone, have a very dramatic effect on the cell by flushing (emptying) most of its cellular contents; thus, the information obtained from ultrastructural studies depends on the type of fixation, among other things, and therefore the level of structure conservation [77-79]. In several cell lines it was demonstrated (Fig. 1) that methanol/acetone treatment preserves microtubules but depolymerizes microfilaments and disrupts both mitochondrial and nuclear morphology. PFA-PBS preserves the general cytoarchitecture, but compromises the quality of microstructural detail. PFA-CBS was found to be the best method to fix and preserve cellular microarchitecture (actin, tubulin), without inducing any cellular change.

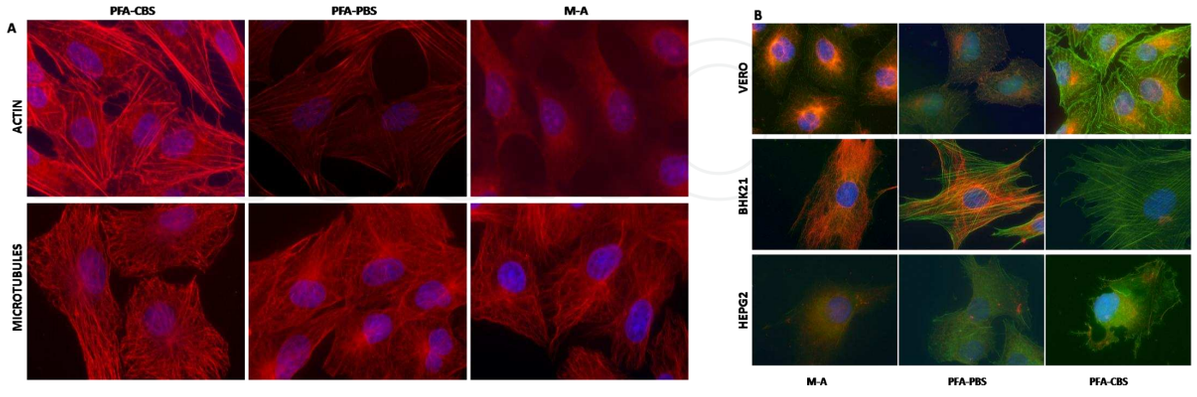

Figure 1. Fluorescence microscopy optimization for visualizing cellular elements. The methanol-acetone (MA) standard protocol does not preserve actin filaments. A comparison between PFA-PBS and PFA-CBS did not demonstrate wide differences in the most notable structures, however, only PFA-CBS method revealed some cellular components which were stabilized satisfactorily. It was found that the optimal concentration for cytoskeletal element visualization was with 3.8% PFA-CBS to fix the cells and antibodies or fluorophores dilutions in CBS of +5% FBS.

2.2. Non-cytotoxic concentrations of cytoskeleton depolymerizing agents

The integrity and functionality of cytoskeleton elements are necessary for their hypothesized role in DENV infections, for which there functionality was evaluated by means of depolymerizing agents. These agents are not entirely harmless to the cells, something which was determined by means dosage ranges having low cytotoxicity (10–20% cytotoxicity) Fig. 2 (a, b). In Fig. 2 (c), the pattern and distribution of cytoskeleton and nucleus components are normal, with no condensation or fragmentation observed. Cellular alterations due to a toxic injury were not evident in these concentrations.

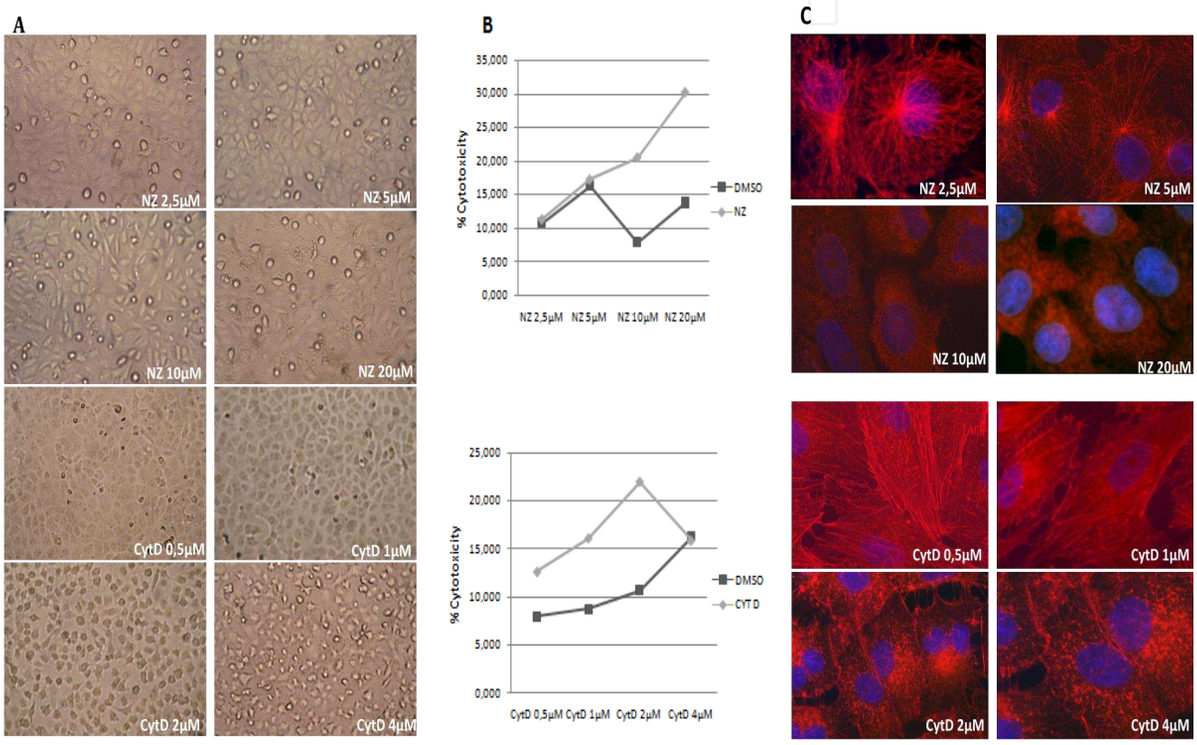

Figure 2. Cytotoxicity assays for cytoskeleton depolymerizing agents. Cytotoxicity assays were performed by cell viability measuring by the method of MTT [3-[4,5 dimetiltiazole-2yl)-2,5 diphenyl tetrazolium bromide), to determine NZ and CytD concentrations that were below the toxic level to cells. We were able to observe the NZ (a, c, e) and CytD (b, d, f) effect in Vero cells. It was found that NZ 10 µM and 4 µM CytD had a cytotoxicity of about 20% and 15%, respectively.

2.3. Relationship between DENV2 and the cytoskeleton

In order to determine some relationship between viral particles and cytoskeletal elements in several steps of the replicative cycle of DENV, Vero cells were infected with DENV2, and processed 18 hours post infection (hpi) by fluorescence microscopy. The fluorescence signal coincident between actin, Fig. 3 (a, b), or the microtubules (c–f), and the viral protein, matching the envelope protein with stress fibers (b) for actin, and microtubules (d), suggests some potential structural relationship, which has an involvement of these cytoskeleton elements within some steps of the DENV infectious cycle.

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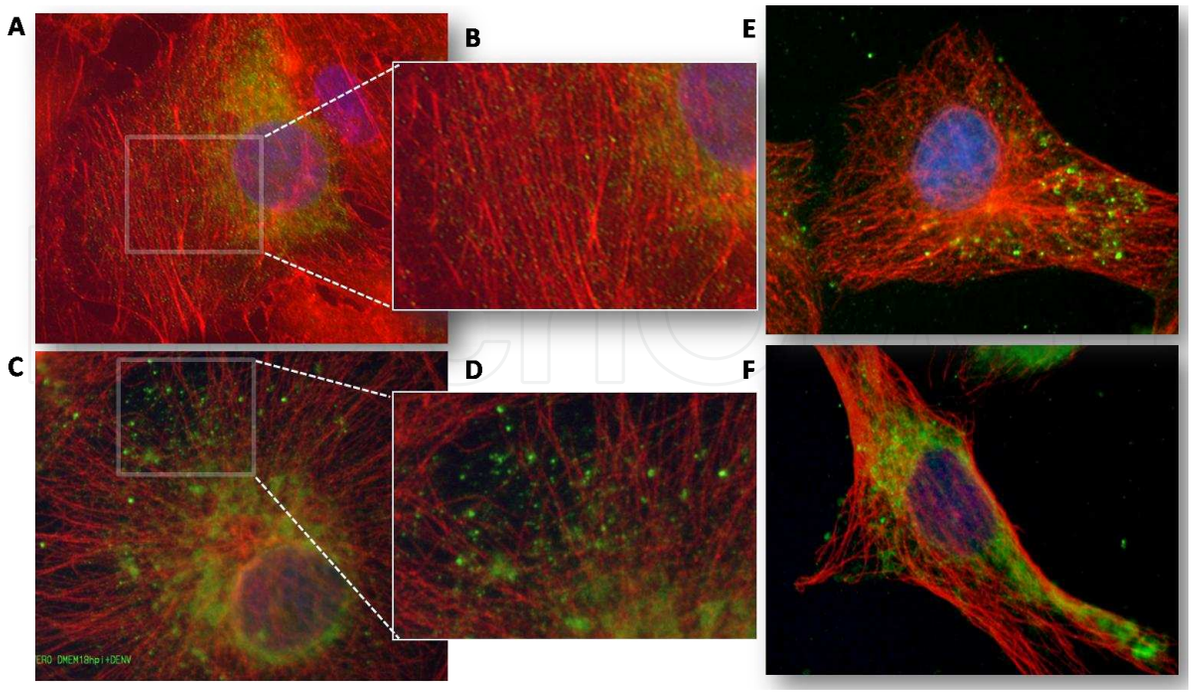

Figure 3. DENV2–cytoskeleton interaction. Vero cell DENV2 infected, viral particles interact with actin (a–b); viral particles interact with microtubules (c–f), were used Phalloidin-Alexa 594 for detect actin or Mab antiβ III Tubulin-Cy3 for microtubules, polyclonal antibody anti-Envelope + antiR IgG Alexa Fluor® 488 for viral envelope protein and Hoechst 33258 for nuclei.

2.4. Subcellular distribution pattern of viral protein altered by depolymerization of microtubules (treatment with nocodazole, NZ)

To investigate the effect of the interrupted cytoskeleton on the viruses replication cycle, Vero cells were treated with NZ before, or after, DENV2 infections. As shown in Fig. 4 (a) and Fig. 5 (a), mock-infected cells without treatment using microtubule depolymerizing agent, NZ, had structures and patterns of observed microtubules traditionally described, i.e., long filaments which are flexible and winding that occupy almost the entire space of the cytosol which emerge from the perinuclear region and are consistent with the MTOC. In contrast, cells that were treated with NZ, Fig. 4 (a), had microtubules which were completely depolymerized, as evidenced by a very fine spotted pattern when observed – something which was not observed the perinuclear region of the MTOC.

Infected cells without treatment with NZ showed a subcellular pattern distribution of diffuse staining of the viral envelope protein, with some small dotted clumps. In order to understand whether microtubules are required for viral entry, treatment was performed 6 hours before infection with NZ (5μM and 10μM), and then with DENV2 at a multiplicity of infection (MOI) of 5 for 1 hour. The inoculum was retired, washings were performed with PBS, and cells were fixed at 24 hpi. The results obtained by fluorescence microscopy indicated that there was a great decrease in the number of infected cells, Fig. 4 (a). In contrast to this, when the cells were infected and post-treated with NZ, significant alterations were produced in this distribution pattern, showed the envelope protein in vesicles as being distributed throughout the cyto-

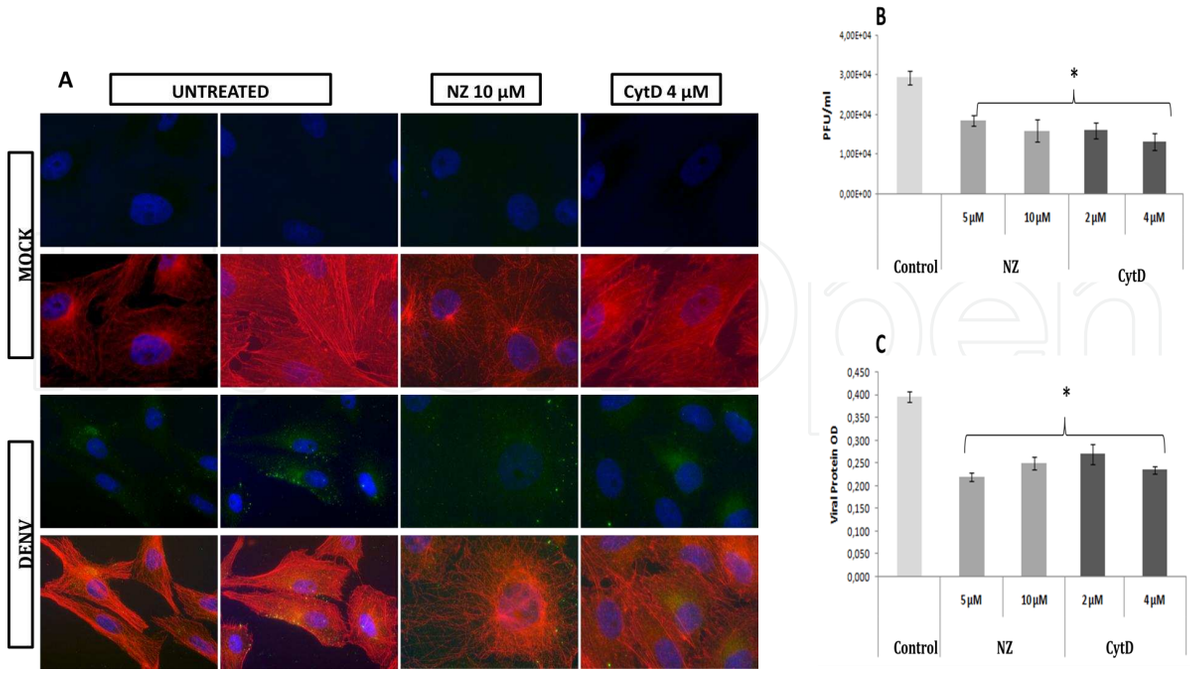

Figure 4. Effects of treatment with NZ and CytD prior to DENV2 infection Vero cells were treated with NZ 5 at 10 µM and CytD 2 at 4 mM, 6 hours before infection to determine if these elements were necessary for virus entry. In both treatments a decrease in infected cells was observed using fluorescence microscopy (a). For NZ and CytD there was an inhibition of about 50% in PFU compared to the control (b), and also a significant decrease of viral protein (c). Were used monoclonal anti-β III Tubulin-Cy3 to detect microtubules, Phalloidin-Alexa 594 for detect actin, polyclonal anti-Envelope + antiR IgG Alexa 488 for viral envelope protein and Hoechst 33258 for nuclei.

plasm, with an apparent subcellular distribution compatible with a Golgi complex Fig. 5 (a). This is because microtubule depolymerization–induced NZ treatment leads to Golgi apparatus disintegration, since the DENV maturation processes used the secretory route in this organelle. In all treatments with NZ, the nucleus was observed with a normal structure.

2.5. Subcellular distribution patterns of viral protein altered by actin depolymerization (treatment with CytD)

To investigate the effect of the interrupted cytoskeleton on the virus replication cycle, Vero cells were treated with cytochalasin D (CytD) before or after infection with DENV2. The mock-infected cells without the depolymerizing agent, CytD, showed a normal pattern with long and parallel fibrils distributed throughout the cytosol, mainly stress fibers, Fig. 4(a) and Fig. 5 (a). This is dramatically reduced after treatment with CytD, resulting in actin depolymerization, and leaving it in a spotted pattern of coarse grains, producing some larger groupings. In the case of actin microfilaments, in DENV2 infected cells, somewhat different findings arose compared with those observed with microtubules. In cells without CytD treatment, remodeling of actin microfilaments was clear, where cells were characterized by *filopodia* formation and many cells were observed with actin tails. These two actin conformations may be related to actin remodeling by Rho GTPases signaling pathway effectors. The envelope viral protein showed two subcellular distribution patterns, one being a diffuse pattern which was very fine

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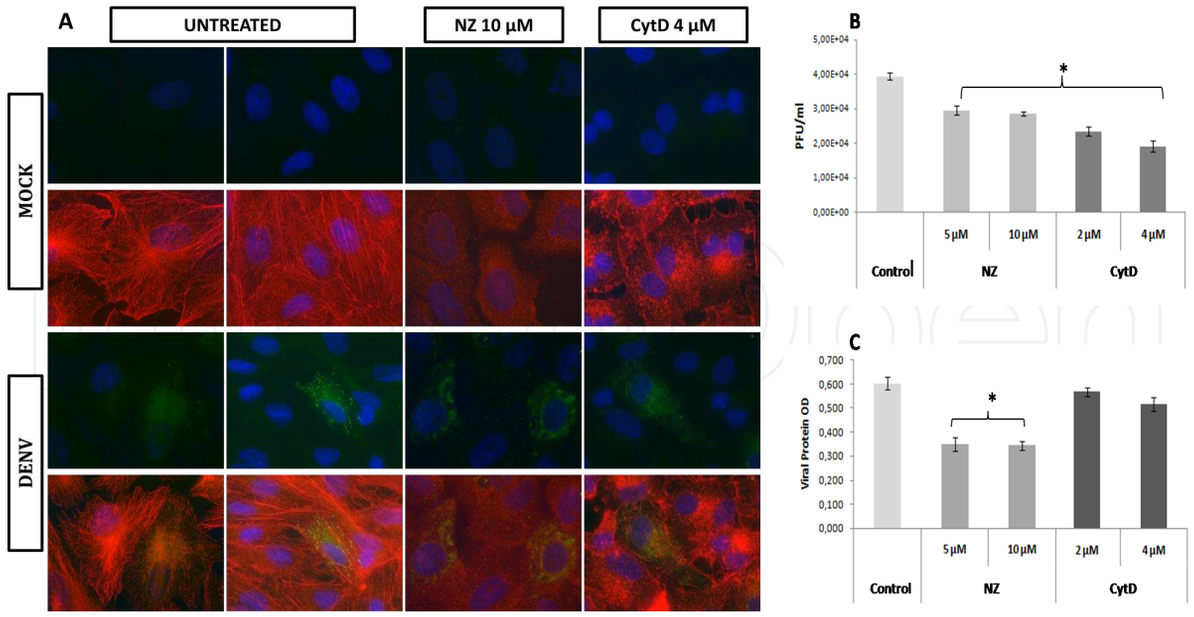

Figure 5. Effects of treatment with NZ and CytD after DENV infection. Vero cells were infected with DENV for 1 hour, the inoculum was removed and washed with PBS, after this the cells were treated with NZ 5–10 µM and CytD 2–4 mM, for 24 hours, in order to determine if these elements were necessary during the replicative cycle of DENV. In both treatments there was a decrease in infected cells when viewed using fluorescence microscopy (a), which was confirmed by the reduction of PFU in the supernatant of the treated cells (b) and viral protein concentration (c). Were used monoclonal anti-β III Tubulin-Cy3 to detect microtubules, phalloidin 594 for actin, polyclonal anti-Envelope + antiR IgG Alexa 488 for viral envelope protein and Hoechst for nuclei

and distributed in the perinuclear region throughout the cytosol, while the other pattern showed a few small clumps of viral protein. According to previous findings in vaccinia virus [45] and DENV [45, 65] the first patters is called "non-assembled viral protein" and the second "assembled viral protein."

In order to understand actin's role in whether microtubules are required for viral entry, the treatment was performed 6 hours before infection with CytD (2µM and 4µM) then with DENV-2 at a MOI of 5 for 1 hour. The inoculum was retired, washings were performed with PBS, and cells were fixed at 24 hpi. To depolymerize actin filaments with CytD in DENV2 infected cells, the subcellular distribution pattern of these cells was altered similarly to uninfected cells. The results obtained by fluorescence microscopy indicated that there was a decrease in the number of infected cells, Fig. 4 (a), with observations using fluorescence microscopy showing a clear accumulation in the perinuclear region that matched the viral factory. In contrast, when cells were infected and post-treated with CytD, significant alterations in this distribution pattern were produced, although the two patterns of distribution of "assembled" viral protein and "non-assembled" changed very little, since they remained in both cells infected with DENV2 and treated with CytD, and in those infected without CytD, Fig. 5 (a). Nevertheless, it is notable that the amount of viral protein "assembled" was markedly reduced in CytD treated cells, suggesting the great role of actin integrity in viral assembly. In all treatments with CytD, the nucleus was observed with a normal structure, Fig. 5 (a).

2.6. The dynamic integrity of microtubules necessary to maintain efficiency of viral replication

To assess if microtubule dynamic integrity has any functional impact on the viral replication process, DENV2-infected cells were pre- or post-treated with 5μM and cell ELISA, after which supernatants were collected in order to quantify viral infectious particle production and intracellular viral protein. NZ pre-treatment, in addition to causing a decrease in the number of infected cells, also produced a decrease in the number of plaque forming units (PFU) quantified in the supernatant with respect to a control group, where the percentage of inhibition od PFUs was 37% for NZ 5 μM and 46% for 10 μM, Fig. 4 (b), and the amount of viral protein in infected cells was reduced to 43% and 37%, respectively, Fig. 4 (c). NZ post-treatment produced a decrease in the number of infectious particles with respect to the control group (infected cells, DMSO), measured by plaque assay (PFU), between 25% and 28% for 5μM and 10uM of this depolymerizing agent, Fig. 5 (b), consistent with a decreased viral protein of 42% approximately for both NZ concentrations, Fig. 5 (c). Together, these results suggest a role is played by microtubules in the assembly, and export, of infectious viral particles. It is probable that, if cellular structures are not properly preserved, not possible observe changes associated with the infection. In this work, we note that altering microtubule dynamics has a direct effect on infection with DENV2, contrary to what was reported by [60].

2.7. Integrity of actin filaments has a greater role in viral protein production than microtubule integrity

To assess if actin dynamic integrity has any functional impact on the viral replication process, DENV2-infected cells were pre- or post-treated with 2μM and 4mM of CytD, after which supernatants were collected for quantifying the viral infectious particle production, and cell ELISA measured intracellular viral protein expressed. In CytD 2–4 μM pre-treated cells, a clear diminution of viral infectious particles was observed, 35–43%, Fig. 4 (b), and viral protein 47–56%, Fig. 4 (c). Interestingly, CytD post-treatment produced a significant decrease in the number of infectious particles production with respect to a control group (infected cells, DMSO), which were measured by plaque assay (PFU), detecting a reduction in viral titer of approximately 37% for CytD 2 μM and 50% for 4 μM, Fig. 5 (b). Actin filament depolymerization produced an insignificant decrease in the amount of viral protein, with non-statistical differences with respect to the control cells, Fig. 5 (c). This suggests that actin may be involved not only in virus entry, but also in any step of virus maturation and, since depolymerized can alter the assembly of viral infectious particles and/or delay the output of these, reflected in a decrease of PFUs and in an intracellular accumulation of viral protein. Thus, although the depolymerization of microtubules and actin filaments reduces the production of infectious viral particles, actin filaments appear to have the most relevant role in this process.

Most recently authors found out that the cytoskeleton is not really necessary for diverse virus replication, specifically for these viruses: Sindbis virus (SINV; family Togaviridae), vesicular stomatitis virus (VSV; family Rhabdoviridae), and Herpes simplex virus (family Herpesviridae) [80]. Although these interesting data suggest a non-conventional role of the cytoskeleton, it must be taken into account that the images are only partially showing the phenomena,

because the fine elements of the cytoskeleton (i.e., actin and microtubules), apparently demonstrated some artificial effects due to their preservation/fixation conditions.

There is an important role played by cytoskeleton elements in the assembly and morphogenesis of several viruses, reported in classical works [81] and more recent papers [30, 82, 83], which partially explains the data shown here. The replication complex and the viral factories are cellular structures from viral origins, but these complex structures need macromolecular and cellular constituents. In viral factories membranes and cytoskeleton elements are recruited as part of their constituents, because it is necessary to limit viral activities in this limited space [32]. In this sense, it is logical to attribute the important role of actin and microtubules in the viral assembly process, which in the case of some kind of blocking might produce a reduction in viral particle production.

Although there are some works about the role of the cytoskeleton on DENV infection, in which the participation of filopodia in DENV entry, as well as the function of Rho GTPases in regulation, is confirmed – a process by means of a cross-talk between Rac1 and Cdc42 [74, 76] – the preservation/fixation techniques in those specimens were not the best. Therefore, there are some doubts about the real participation of these elements in the dengue viral infections, because once the cells are significantly damaged the possibility for making the ulterior steps of the infectious cycle are difficult. However, the early stages of viral infections, like those shown by means of filopodia, are undoubtedly because the fine elements are well preserved.

In this vein, there is a collection of data, via images, proteins, and production of virions, demonstrating that the cytoskeleton plays an essential function in the viral replication processes. The viruses can be considered as probes for dissecting cellular signaling, cytoskeleton reorganization, and endocytosis [83, in this sense the research with dengue viruses could be considered similarly – with the actin microfilaments used for the viral assembly process since the disruption of these elements causes envelope viral protein aggregation. Images of "non-assembled" viral proteins have not been shown in others works. This pattern described here is compatible with other viruses [45, 84]. One such piece of work shows that altering actin remodeling by inhibition of a HMG-CoA reductase produces a scattered punctuated fluorescence pattern in viral protein after the treatment of lovastatin, which is compatible with an impaired assembly process. In agreement with these findings, we have shown the DENV2 infected cells treated with lovastatin, produce a clustering of envelope viral protein which also is attributed to some difficulties in the viral assembling process [65].

Consistent with this interpretation, the amounts of viral protein which had accumulated after the inhibition of actin filaments with cytochalasin D treatment was higher in comparison with the microtubules inhibited in the same conditions. If the "non-assembled" protein is reflecting some difficulty in this case [45, 65, 84], the potential role of actin microfilaments is implied in the viral assembly and/or maturation process of DENV. The indirect connection of actin microfilaments, Rho GTPases, with DENV assembly [65] had been used for clinical trials [85], based on the pleiotropic effects of inhibition of HMG-CoA by means of lovastatin [66]. The findings shown here offer new perspectives in the viral replication and morphogenesis of DENV, since at present there is lack of knowledge about the role played by actin in the viral factories and/or in the replication complex. On the other hand, the advantages of the technical

approach shown here have been evidenced with other work, in which curcumin is able to alter the DENV2 replication process by blocking several cellular processes such as ubiquitin-proteasome and those of the cytoskeleton [72].

In spite of the diverse roles microtubules and actin microfilaments have in the different viral steps of replication for diverse viruses, more recently it was found that the replicative complex has intermediate filaments of vimentin which have a close, and structural, relationship with the non-structural protein 4A of DENV, forming part of their replication complex [86].

3. Conclusions

There has been an increased interest in recent years in understanding the cellular mechanisms that viruses exploit in the host cell, "Cell Biology of Viral Infection" is a new discipline that seeks to understand these intricate mechanisms [1]. Hence, in order to describe the participation of the cellular elements in DENV2 infection it is necessary to preserve the fine structure of cell morphology as closely as possible by avoiding any generation of artifacts.

In this work we have demonstrated the involvement of the cytoskeleton during DENV replication. It was determined that depolymerized microtubules and actin microfilaments generate disturbances in the DENV2 lifecycle, which causes a reduction in the production of infectious viral particles and in the intracellular expression of the viral envelope protein, as well as an altered subcellular distribution pattern of the viral protein envelope. This effect was more significant in the depolymerization of microtubules that in actin microfilaments. It has been reported that viral infections alter the global host proteomic in response to these, including cytoskeletal proteins [87-89]. However, although a role for the involvement of these proteins in viral infection is hypothesized, a route through which this occurs has yet to be identified.

4. Methods

4.1. Cell lines and virus

Aedes albopictus mosquito C6/36HT cells and epithelial Vero cell line (American Type Culture Collection CCL-81™) were grown in Dulbecco's Eagle's minimum essential medium (DMEM, GIBCO) supplemented with 2% or 10% fetal bovine serum (FBS, GIBCO), with 100 U/ml penicillin/100 μg/ml streptomycin and 0.25 μg/mL Amphotericin B. The cells were grown at 34°C with 5% CO_2 (C6/36HT) and 37°C with 5% CO_2 (Vero). DENV2 (New Guinea strain) was generously donated by María Elena Peñaranda and Eva Harris from the Sustainable Sciences Institute (SSI), San Francisco, California. The viruses were propagated in C6/36HT cells at low multiplicity of infection (MOI: 0,01 PFU/cell) and stored at -80ºC until used. Viral titers were detected by plaque assay, using a Vero cell monolayer culture under 1% methylcellulose overlay medium.

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4.2. Antibodies and chemicals

The serum from rabbits against the envelope protein of DENV (polyclonal anti-DENV) was donated by Dr. Eva Harris and Robert Beatty (University of California, Berkeley, CA, USA). The secondary antibody coupled to peroxidase and anti-β-tubulin monoclonal coupled to Cy3 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Mycotoxin phalloidin conjugated with Alexa Fluor-488 or Alexa Fluor 594 and acetylated tubulin were purchased from Molecular Probes Invitrogen Life Technologies (Carlsbad, CA, USA). CytD, NZ, and 3-(4,5-dimethyl thiazol-2yl)-2,55-diphenyltetrazolium bromide (MTT) were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). FluorSave was purchased from Calbiochem.

4.3. MTT assay

Cell viability was measured by MTT assay [90] to determined NZ and CytD concentrations that were below cytotoxic levels. Cells were seeded, 25,000 cells/well, in 96-well plates and allowed to adhere for 24 hours at 37ºC in 5% CO_2; cells were exposed to MTT (0.5mg/ml) for 4 hours, acid isopropanol (100µl) was then added to solubilize the formazan crystals produced. The culture dishes were incubated for 30 minutes and the absorbance was measured at 570nm in a Benchmark reader (Bio-Rad Laboratories, Hercules, CA). Vero cells were treated with 6 concentrations of these cytoskeleton-depolymerizing agents (NZ: 1.25, 2.5, 5, 10, 20 and 40µM) and (CytD: 0.25, 0.5, 1, 2, 4 and 8µM) for several periods (3, 6, 12, and 24 hours). The NZ and CytD were chosen at two concentrations (5µM and 10µM) and (2µM and 4mM) with a lower cytotoxicity (10% and 15%), but a preserved ability to depolymerize microtubules and inhibit actin polymerization, respectively. These agents were added to cultures 1 hour post-infection (hpi) with DENV.

4.4. Viral infection and treatments

In order to determine the cytoskeletal depolymerizing agent's effects on DENV2 replication, experiments were undertaken as follows. Confluent Vero cell monolayers were grown on 24 (1×10^5) and 96 (2.5×10^4) multi-wells, in DMEM with 2% FBS, at 37°C and 5% CO_2. Cells were infected in the absence of FBS at an MOI of 5 PFU/cell, after a 1-hour adsorption period at 4ºC, the viral inoculum was replaced with maintenance DMEM and incubated at 37ºC. Infected cells were incubated in the presence of NZ, CytD, or DMSO for the indicated times. These cells were maintained for 24 hours after infection and treatment. As a cytoskeleton infection control, we performed the same procedure without DENV under the same conditions. Coverslips for immunofluorescence (IF) were placed in 24 multi-wells. The cell monolayers were fixed and processed to visualize the viruses and cytoskeleton components by confocal fluorescence microscopy. Monolayers and culture supernatants were collected to determine the effect of CytD and NZ in viral protein production by cell ELISA. Additionally, the supernatants also were used to quantify the cytoskeleton blockage effect in infectious viral particle production by means of titration by plaque assay.

4.5. Titration by plaque assays

For quantification of infectious viral particles, the virus was titrated by plaque assays using supernatants of Vero cells infected with DENV2 and treated under different conditions, as described in [91, 92]. Briefly, cells were plated, $5x10^4$ cells/well, on 24-well plates, in DMEM 2% FBS at 37°C in 5% CO_2, and inoculated with serial dilutions of viral collected supernatants from 10^{-1} to 10^{-5} in DMEM without FBS. At 1 hpi the inoculum was removed, washed, had DMEM 2% FBS and finally 1.5% of carboximetilcelulose (SIGMA) added to it. After 8 days post-infection (dpi), the cells were fixed with paraformaldehyde (PFA) at 4% in PBS and revealed with crystal violet. The plaque number was recorded in order to calculate the PFU/ml. Two independent experiments were performed, each in duplicate.

4.6. Cell ELISA

Viral protein was quantified by a spectrophotometric cell ELISA, which is a modification of fluorometric ELISA previously described by [65]. To this immunodetection of viral protein in infected cells, Vero cells ($2.5x10^4$ cells) were submitted to different experimental strategies and were fixed for 30 minutes with 4% paraformaldehyde (PFA), washed three times with PBS, and permeabilized for 30 minutes with 0.1% Triton X-100. Endogenous peroxidase was quenched with 0.3% H_2O_2 in 10% methanol and, non-specific sites were saturated with a blocking buffer (10% FBS in PBS). Then the anti-DENV2 diluted (1:500) blocking buffer was incubated for 1 hour at 37°C. After washing with PBS, this was incubated for 30 minutes with a secondary antibody, anti-mouse IgG conjugated with HRPO. Finally after washing with PBS, chromogenic substrate SIGMA ® FAST OPD (St. Louis, MO, USA) was added to reveal the reaction and its absorbance was read at 405nm in the Benchmark reader (Bio-Rad Laboratories, Hercules, CA, USA). To normalize the data, the total protein concentration in each well was determined by Bradford assay, interpolating absorbance data in a calibration curve with known concentrations of bovine serum albumin. Two independent experiments were performed with 3 replicates for each assay.

4.7. Fluorescence microscopy

In order to use the best preservation and fixation protocol for assays linked to the research, three fixations were assessed: paraformaldehyde 3.7% in phosphate buffered saline (PFA-PBS), paraformaldehyde 3.7% in cytoskeletal buffered sucrose (PFA-CBS), and Methanol-Acetone 1-1 (M-A). Vero cells were cultured in 24 multi-well plates with glass cover slips, at a density of $4.5x10^4$ cells/well in DMEM 2% FBS. After 24 hours monolayers were fixed with different treatments. For the PFA-CBS treatment the medium was discarded, washed with CBS at 37°C, and fixed with PFA-CBS over 30 minutes at 37°C in 5% CO_2. For the PFA-PBS treatment the medium was discarded, washed with PBS, and fixed with PFA-PBS over a period of 30 minutes. For the M-A treatment the medium was discarded, washed with PBS, and fixed with methanol-acetone over a period of 30 minutes at 4°C.

To evaluate the cytoskeleton blocking effects of CytD and NZ in DENV-2 infection, 24 hours after treatment Vero cell monolayers were rinsed once at 37ºC with CBS [10 mM PIPES (pH

6.9) (1,4-piperazinebis-ethane sulfonic acid), 100 mM NaCl, 1.5 mM MgCl2, and 300 mM sucrose. The cells were then permeabilized and simultaneously fixed with PFA-CBS for 30 minutes at 37ºC. To avoid auto-fluorescence the cells were placed in 50mM NH^4Cl for 10 minutes, then were permeabilized with 0.3% Triton X-100 and had their non-specific sites blocked with 5% FBS in CBS. For detection of viral protein and cytoskeleton, cells were incubated with primary monoclonal anti-DENV antibody (1:500) and Alexa 488 conjugated secondary antibody. After that, cells were incubated with anti-phalloidin 594 or anti-β tubulin Cy3 and Hoechst 33258. Following washing with CBS, slides were mounted with mounting medium and examined under a confocal fluorescent microscope (Olympus IX-81 DSU). The micrographs were recorded using an Olympus CCD camera and processed with Image Pro Plus software (from Media Cybernetics).

4.8. Statistical analysis

Data are presented as means ± SD. In all cases, they represent at least three independent determinations. The significance of the results was calculated by Student's t test. In this case, p values < 0.05 were considered significant with respect to controls.

Acknowledgements

This research was supported by COLCIENCIAS grant 111554531592 from the Colombian government. JCGG was the recipient of a Full-Time Professor Program (Exclusive Dedication) for the Medicine Faculty at University of Antioquia for 2014–2015.

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The authors declare that they have no competing interests.
AITC contributed to the experimental design, carried out the experiments. EOG worked on analysis interpretation of data, and drafted the manuscript. JCGG conceived the study, participated in its design, and coordinated and finalized the manuscript. All the authors read and approved the final version of the manuscript.

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