International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.6, pp 1933-1940, Oct-Nov 2014

The Effect of Genistein on the Fibroblast R445H OPA1

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Abstract: The mitochondria are an essential cytoplasmic organelle that provides most of the energy necessary for eukaryotic cell physiology. Mitochondrial structure and functions are maintained by proteins of both mitochondrial and nuclear origin. These organelles are organized in an extended network that dynamically fuses and divides. Mitochondrial morphology results from the equilibrium between fusion and fission processes, controlled by a family of mitochondria-shaping proteins. It is becoming clear that defects in mitochondrial dynamics can impair mitochondrial respiration, morphology and motility, leading to apoptotic cell death in vitro and more or less severe neurodegenerative disorders in vivo in humans.
Dominant optic atrophy (DOA) is characterized by retinal ganglion cell (RGC) degeneration leading to optic neuropathy. A subset of DOA is caused by mutations in the OPA1 gene, encoding for a dynamin related GTPase required for mitochondrial fusion. The functional consequences of OPA1 mutations in DOA patients are still poorly understood. OPA1 encodes an intra-mitochondrial dynamin, involved in inner membrane structures and ubiquitously expressed, raising the critical question of the origin of the disease pathophysiology. Mutations in OPA1 somehow interfere with the function of the respiratory complex in the mitochondria reducing the amount of energy produced and increasing the amount of derived reactive-oxygen species (ROS). OPA1 is predominantly expressed in the retinal ganglion cell layer; hence those cells are specifically affected despite the ubiquitous expression of the OPA1 gene in all organs. Moreover RGCs are essentially highly susceptible to the ROS. In this review we investigated whether Genistein has protective effects on damaged mitochondria because of it is own properties as an antioxidant and how it is organizing the mitochondrial network to the extent that the energetic pools of ATP would be evenly spread in the cell. Experiments took place on skin fibroblasts cells OPA1 mutated (R445H), obtained from patients with optic atrophy and these cells had been treated with staurosporine to induce cell death. We then describe the remarkable mitochondrial network physiology and reactions after treating with Genistien at the molecular level as studying the changes occurred on the network and membrane potential after the treatment. Genistein might affect positively on vital factors in mitochondria yet, further studies are needed to explore the exact signaling pathway(s) by which Genistein protects mitochondria
Keywords : Mitochondria, Optic atrophy, DOA, OPA1, Genistien.

Introduction

Mitochondria are eukaryotic subcellular organelles which are commonly referred as the powerhouse of the cell, since they produce most of the cell's energy supply. These organelles, however, are the main players in many other cellular processes, not only the energy production, but also thermogenesis, apoptosis, reactive species of oxygen production and calcium homeostasis.

Mitochondria are unique among the cytoplasmic organelles becuase they contain multiple copies of their own DNA, which encodes a small number of proteins, essential for their energetic function [1]. However, nuclear genes encode the majority of the proteins that are fundamental for mitochondrial physiology, including those responsible for expression and maintenance of the mtDNA.

Formally, the term 'mitochondrial dynamics' includes all of the different ways that the geometrical features of mitochondrial networks change over time. More commonly, 'mitochondrial dynamics' refers to the fission and fusion dynamics that constantly remodel the mitochondrial network. Fission cuts a tubule into two while fusion can link two tubules together to form a longer tubule or a branch. The balance of fission and fusion is important for shaping mitochondrial networks; more fission than fusion leads to over-fragmented networks while more fusion than fission leads to over-connected networks compared to normal, wild-type networks[2, 3].

The critical importance of this organelle is highlighted by the escalating number of human disorders that in recent years have been shown to be due to mitochondrial dysfunctions. Many rare genetic disorders are caused by mutation either in mitochondrial DNA or in nuclear genes encoding for mitochondrial proteins. Mitochondrial dysfunction has also been implicated in more common human diseases, such as several degenerative diseases, cancers, diabetes, and even in the natural process of aging[4-7].

DOA is a neurodegenerative disease which is believed to be the most common of the hereditary optic neuropathies. This disease is the most common form of inherited optic neuropathy, and the second leading cause of blindness in the world[8]. DOA, also known as Kjer's optic atrophy, affects retinal ganglion cells and the axons forming the optic nerve, leading to progressive visual loss[9].

Fibroblasts from DOA patients possess either normal or fragmented mitochondrial networks in comparison with controls[10-12]. Mitochondrial structure alterations have been frequently reported in fibroblasts[10, 11, 13, 14], in myotubes[15], in skeletal muscle from DOA patients[14] as well as in OPA1 mouse models[16].

The most common form of DOA is caused by mutations in the OPA1 gene. OPA1 encodes an intra-mitochondrial dynamin, the nature of this protein demonstrated that DOA is a mitochondriopathy[8].

The OPA1 gene is 6031 nucleotides long and is composed of 31 exons spanning >114 kb of genomic DNA. According to the eOPA1 database, 205 OPA1 gene mutations have been identified, which are basically family specific[17]. These mutations are spread throughout the protein, but the GTPase domain (32,8%), dynamin domain (18,1%), the C-terminus (18,1%) and the N-terminus region (6,9%) are more frequently affected.

The systematic molecular screening of OPA1 in patients with DOA has revealed a wide range of phenotypic variations of the disease. Specific OPA1 mutations are responsible for several distinct clinical presentations in DOA patients, in DOA with deafness (DOAD), and in severe multi-systemic syndromes, the so-called "DOA plus" disorders [14, 18].

From the observations, two hypotheses arise that can link mutations in OPA1 and mitochondrial dysfunction. One is that truncated or partially inactivated proteins might somehow destabilize the mitochondrial inner membrane and therefore interfere with the function of the respiratory complexes, reducing the amount of energy produced and increasing the amount of derived reactive-oxygen species (ROS). The second hypothesis is that mutated proteins might partially disorganize the mitochondrial network to the extent that the energetic pools of ATP would be unevenly spread in the cell[8].

Despite the ubiquitous expression of OPA1 gene in all organs; retinal ganglion cells (RGC) are specifically affected by mutations in OPA1. One possibility reasons is that OPA1 is predominantly expressed in the retinal ganglion cell layer[19]. Moreover retinal ganglion cells are fundamentally highly susceptible to the reactive-oxygen species[20]. Vulnerability of retinal ganglion cells could also be related to specific requirements in the distribution of the energy along their axons and lead to the failure of the transport in the optic nerve. Another hypothesis is that the optic nerve mitochondrial network in necessary to maintain transmission of action potential along the axon and particularly in the non-myelinated laminar proteins which are rich in mitochondria[21].

The R445H mutation is the only OPA1 mutation that has been associated with this unique syndrome of optic atrophy, sensorineural hearing loss, ptosis, and ophthalmoplegia in two unrelated families[22]. Dominant optic atrophy, ptosis, ophthalmoplegia and sensorineural deafness have previously been described in a Belgian and North American family with the R445H OPA1 GTPase-domain mutation[22, 23]. Intriguingly, R445H is one

of the most common OPA1 mutations, but the phenotype usually only involves the visual system [18]. The heterozygous R445H mutation in OPA1 was found in patients with optic atrophy and deafness. Skin fibroblasts showed hyper fragmentation of the mitochondrial network, decreased mitochondrial membrane potential, and adenosine triphosphate synthesis defect. Thus, optic atrophy and deafness may be related to energy defects due to a fragmented mitochondrial network[13].

As mentioned before retinal ganglion cells are specifically affected by mutations in OPA1. Since all derived cells from patients were skin fibroblasts, therefore it was necessary to apply a stress effort on the cells by using staurosporine, where staurosporine is a natural alkaloid. It is widely employed as an inducer of apoptosis in many mammalian cell types[24, 25].

Genistein is a natural compound belonging to the class of isoflavones. While isoflavones are widely distributed in plant Kingdome, the concentration of these compounds is relatively high in particular in soybeans [26,27]. Numerous experiments have been undertaken show that genistein interferes with many biochemical pathways and its mode of action in the live cell is complex and multidirectional. One of the biological activities of genistein explored in cell is antioxidant effects among many other effects[28]. Some studies show evidence regarding the protective effect of Genistien on mitochondrial dysfunction induced by oxidant production[29].

This work aims to studying the effect of Genistein on the Fibroblast R445H OPA1, and characterize the effect of this product on the mitochondrial network and mitochondrial membrane potential.

Materials and Methods:

Cell Culture:

Skin fibroblasts were derived, from DOA patients with the mutation R445H OPA1. These cells were cultured in Dulbecco Modified Eagle Medium (DMEM) (Euroclone) supplemented with 10% fetal bovine serum (FBS) (Euroclone), 2 mmol/L L-glutamine (Euroclone), 100 units/mL penicillin (Euroclone), and 100µg/mL streptomycin (Euroclone), at 37°C in a 5% CO_2 humidified incubator. For incubations in galactose medium, DMEM glucose-free medium supplemented with 5mM galactose (Euroclone), 2 mM L-glutamine, 5 mM Na-pyruvate and 5% FBS was used. Transfer the cells from the growing flask to wells and prepare them to the treatment. By using a counting chamber determine the desired volume to seed.

Cell Viability Test: MTT after treating with Genistein:

Test performed in 96 wells plate after transferring the required number of cells to the wells (2500 cell/well). Different dilutions were prepared and applied separately of Genistein diluted by DMSO: 0.1µM, 0.3µM, 1µM, 3µM, 10µM and 30µM. Incubated for 24hours in 37°C. Apoptotic stress was triggered by using staurosporine (STS) (SIGMA-ALDRICH) 1.75µM for 3 hours in 37°C. For positive control add STS diluted with the medium to the same concentration. For the negative control add appropriate volume of DMSO and medium. For cell viability test MTT (SIGMA) 5mg/ml: DMEM was used with incubation period for 3 hours in 37°C. Spectrophotometrically measure absorbance at a wavelength of 570nm-655nm is applied using Bio Rad machine and microplate manager as a soft ware analysis.

Mitochondrial Network Structure (Mitotracker) after treating with Genistein:

MitoTracker is applied according to Invitrogen manual and protocol sheet to stains mitochondria in live cells then recognize the mitochondrial network phenotypes of 100 cells. To label mitochondria, cells are simply incubated with MitoTracker probes 100 nM (Invitrogen) for 3 hours in 37°C after treating with Genistein 3µM and after apoptotic stress caused by staurosporine 2µM. mtDNA and nuclear DNA were labelled by adding 0.5 µl DAPI (SIGMA) 3µM. Observed by a microscope and images were captured by a confocal microscope (Zeiss, LSM 510 Meta) with a 40X oil objective (Diaphot, Nikon, Japan) using Metamorph acquisition/analysis software (Universal Imaging Corp., Downingtown, PA, USA).

Quantitative Measurement of Mitochondrial Membrane Potential (JC1) after treating with Genistein:

2 ml of a 5µg/ml JC1 (SIGMA-ALDRICH) was added to 100,000 seeded cells which are treated by Genistein 3µM and stressed by staurosporine2µM and incubated for 30 min in 37°C. mtDNA and nuclear DNA were labelled by adding 0.5 µl DAPI (SIGMA) 3µM. The cells were observed with two different wavelengths of

emission: 538 nm (green) and 590 nm (red). The ratio 590/538 provides the mitochondrial membrane potential in relation to mitochondrial mass.

Results:

Cell Viability Test: MTT after treating with Genistein:

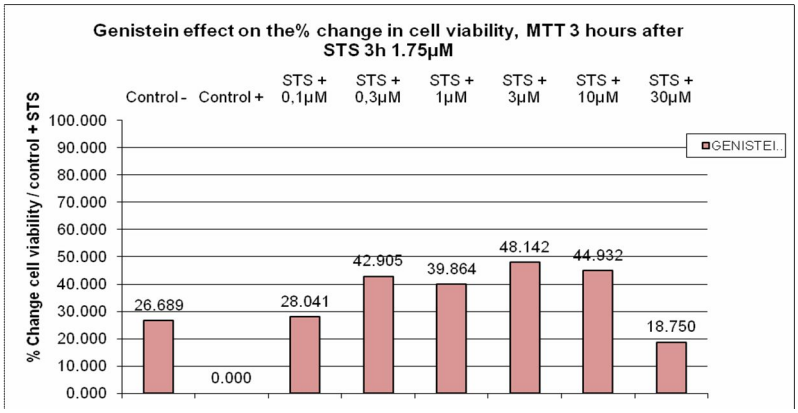

Figure 1: Effect of Genistein on cell viability percentage after applying apoptotic stress caused by STS. Results in figure are indicating the average of two times experiment

Genistein 3µM significantly promoted the cell viability compared with the other concentration in the experiment, consequently of the results in (figure 1) indicate an increase in the percentage of cells viability in this concentration; all further experiments to measure or test the membrane potential and network structure of the mitochondria in cultured cells are applied to study the affect of the product in this concentration (3µM).

Mitochondrial Network Structure (Mitotracker) after treating with Genistein:

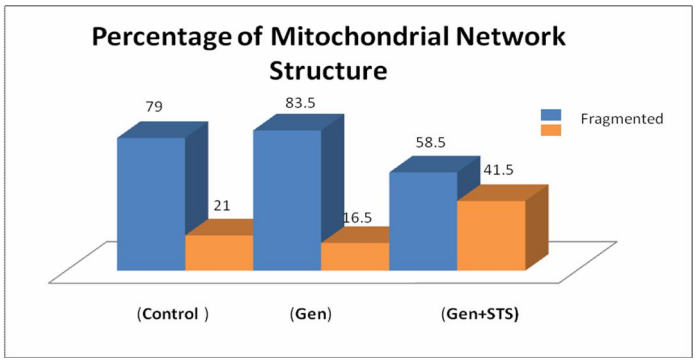

Figure 2: Percentage of different phenotypes of mitochondrial network among 100 cells with each treatment.

Recognize the filamentous phenotype and fragmented phenotype on 100 cells repeated twice. The results indicate that mitotracker is a sensitive indicator of relative changes in mitochondrial network morphology, whereas Genistien is relatively effective on network changes under apoptotic stress caused by staurosporine, as the results showed in (Figure 2) we can notice the notable changes in the percentage of cells with filamentous network after treating with both Genistien and staurosporine. Yet there is no significant changes can be mentioned caused by the lone effect of Genistien its self compared with the control. (Figure 3) shows samples of morphologies concerning mitochondrial network from DOA fibroblasts.

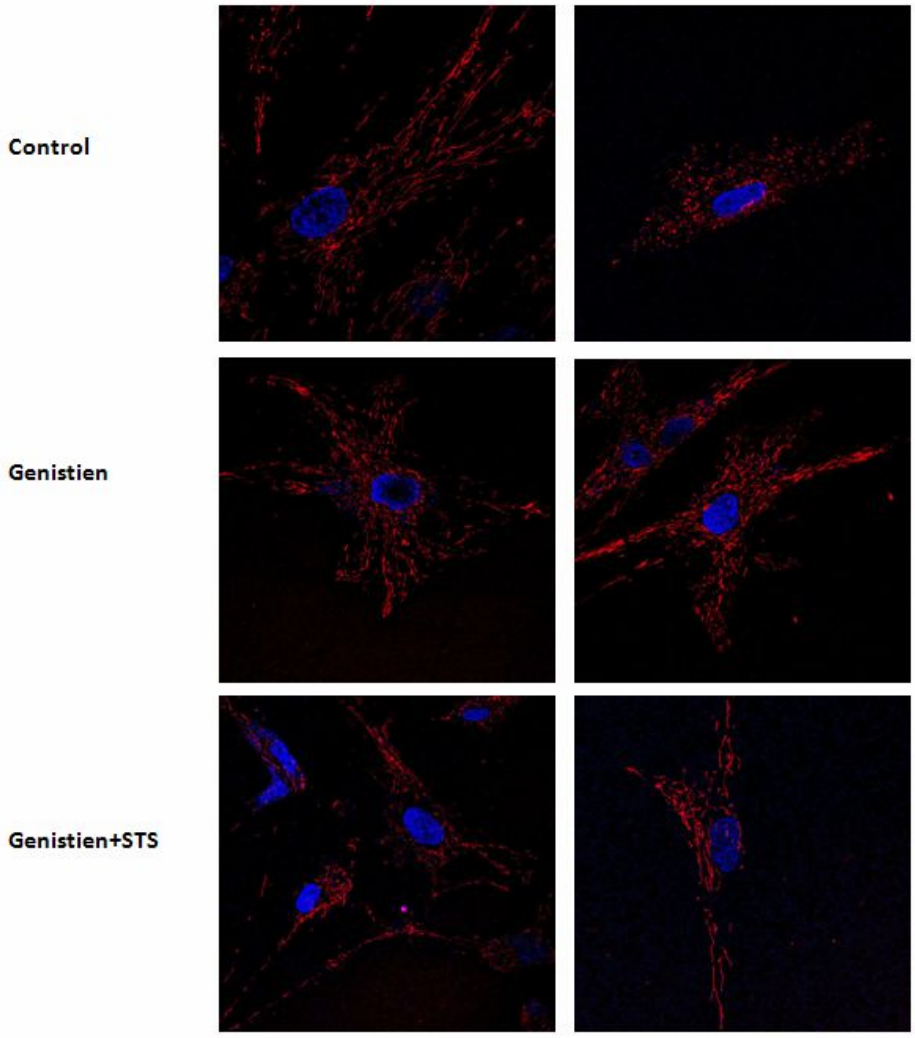

Figure 3: Representative images of mitochondrial morphologies of DOA fibroblasts incubated and captured with different treatment to indicate any recognizable changes in mitochondrial network.

Quantitative Measurement of Mitochondrial Membrane Potential (JC1) after treating with Genistein:

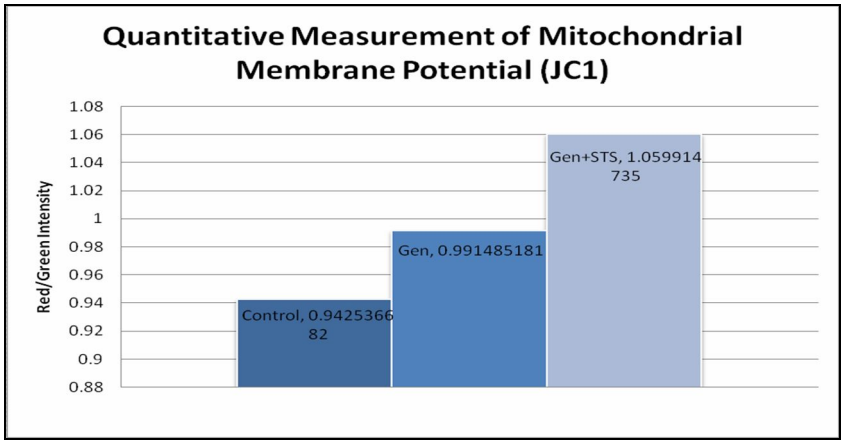

Figure 4: Quantitative results of mitochondrial membrane potential after treating with Genistein and staurosporine.

Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The potential-sensitive colour shift is due to concentration-dependent formation of red fluorescent aggregates; where a higher red means a high membrane potential while a higher green means a low membrane potential. (Figure 4)

These results show a slight changes on the comparative measurements of membrane potential when treated by both Genistein and staurosporine, in other hand those results do not reasonably determine the percentage of mitochondria within a population that respond to an applied stimulus by Genistein.

Discussion

Mitochondria are the major organelle of essential energy production for cell activity, and have been suggested to be involved in many important physiological activities[30, 31]. The mitochondrial respiratory chain is a major site of ROS production in the cell, and thus mitochondria are suggested as a prime target for oxidative damage [32]. In a physiological state, most mammalian cells keep a balance between the generation of ROS and its removal. However, when the mitochondria suffer from injuries this balance is likely broken resulting in oxidative stress that leads to cell death[30-34].

Data of previous studies have indicated that Genistein as a dietary antioxidant plays a protective role in neurodegeneration[35-40].

Intracellular oxidative stress is also known to alter a cell's mitochondrial $\Delta\Psi m$[41]. Researches focused on mitochondria of PC12 cells and found Genistein maintained the mitochondrial redox system disordered by beta-amyloid peptide 25–35 (Aβ25-35) and then maintained mitochondrial membrane potential.

Based on the present results that Genistein maintains mitochondrial membrane potential and redox system in mitochondria and PC12 cells[38, 40], we speculate the data obtained in our study show that Genistein pretreatment considerably increases the viability percentage of fibroblast cells obtained from DOA patients with a mutation on OPA1 gene, and affect positively on other vital factors in mitochondria. Further research is needed to clarify the relationship between mitochondrial dysfunction and OPA1 mutations, and the mechanism of Genistein to protect mitochondrial systems and functions. However, pretreatment with Genistein might reverse the damage of the mitochondrial membrane and increase mitochondrial antioxidant function. Further studies are needed to explore the exact signalling pathway(s) by which Genistein protects mitochondria. Although using fibroblast is recommended in researches because of it's ease of cultivation feature and mutation in all genes encode mitochondrial proteins can be clearly expressed in these cells, but research might be useful on other types of cell expressly on ganglion cells if it's possible. On other hand experiments should be applied in a larger number of cells so statistical results can be more valuable.

Conclusion

Pre-treatment with Genistein might affect positively on vital factors in mitochondria and considerably increases the viability percentage of fibroblast cells obtained from DOA patients with a mutation on OPA1 gene.

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