

RESEARCH ARTICLE

Preconditioning L6 Muscle Cells with Naringin Ameliorates Oxidative Stress and Increases Glucose Uptake

R. Dhanya¹, K. B. Arun¹, V. M. Nisha¹, H. P. Syama¹, P. Nisha¹, T. R. Santhosh Kumar², P. Jayamurthy^{1*}

1 Agroprocessing and Natural Products Division, National Institute for Interdisciplinary Science and Technology (NIIST), CSIR, Industrial Estate, Pappanamcode, Thiruvananthapuram-695019, Kerala, India, **2** Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram-695012, Kerala, India

* jayamurthy@live.com



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Abstract

Enhanced oxidative stress contributes to pathological changes in diabetes and its complications. Thus, strategies to reduce oxidative stress may alleviate these pathogenic processes. Herein, we have investigated Naringin mediated regulation of glutathione (GSH) & intracellular free radical levels and modulation of glucose uptake under oxidative stress in L6 cell lines. The results from the study demonstrated a marked decrease in glutathione with a subsequent increase in free radical levels, which was reversed by the pretreatment of Naringin. We also observed that the increased malondialdehyde level, the marker of lipid peroxidation on induction of oxidative stress was retrieved on Naringin pretreatment. Addition of Naringin (100 µM) showed approximately 40% reduction in protein glycation *in vitro*. Furthermore, we observed a twofold increase in uptake of fluorescent labeled glucose namely 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2 - NBDG) on Naringin treatment in differentiated L6 myoblast. The increased uptake of 2-NBDG by L6 myotubes may be attributed due to the enhanced translocation of GLUT4. Our results demonstrate that Naringin activate GSH synthesis through a novel antioxidant defense mechanism against excessive Reactive Oxygen Species (ROS) production, contributing to the prevention of oxidative damage in addition to its effect on glycemic control.

Introduction

Diabetes mellitus is often defined as a hyperglycemic condition arising due to insulin resistance or impaired insulin secretion. The oxidative stress induced pathways is known to be associated with the onset of diabetes and its complications, which is the real cause of the morbidity and mortality associated with diabetes [1]. Among other factors, oxidative stress is also reported to be responsible for the impaired secretion of insulin and glucose utilization in peripheral tissues in diabetic conditions [2]. Under hyperglycemia, the inhibition of ROS generation or its neutralization is reported to prevent the development of diabetic complications [3–7]. Evans et al.

[8] have also suggested the role of oxidative stress in generating insulin resistance by altering intracellular signaling pathways.

Antidiabetic drugs like metformin are known to ameliorate hyperglycemia by lowering carbohydrate absorption without direct effect on insulin resistance [9–11]. There is a need to look for more efficacious agents for the management of diabetes, owing to the side effects of currently available drugs. Antioxidant treatments have demonstrated beneficial effects in animal models of diabetes [12–14]. However, these effects were not translated in larger clinical trials [15, 16] suggesting the need for new and more efficient antioxidants, targeting multiple factors of diabetes and its complications. For this reason, a potent antioxidant having antidiabetic properties will be a promising candidate for the treatment of both diabetes and its complications. There has been an increase in the use of plant extracts or their active components for health care needs over the last few decades [17]. Among them, Naringin, a flavonone found in grapefruit, has been reported to exhibit various therapeutic and pharmacological properties including antioxidant, antimicrobial, cardioprotective and antimutagenic effects [18]. It has also been reported to decrease blood glucose levels in streptozotocin induced diabetic rats by increasing plasma insulin levels [19]. As pathogenicity associated with diabetes is believed to be due to oxidative damage to the tissues by oxygen free radicals, it is sensible to elucidate the detailed protective mechanism of this potent antioxidant. The aim of this study is to investigate the role of Naringin in glucose uptake and GLUT4 translocation in skeletal muscle cell lines, the major hallmarks in diabetes. In addition, oxidative stress markers, like lipid peroxidation, glutathione, and intracellular reactive oxygen species were assessed as a possible mechanism underlying the Naringin effects.

Materials and Methods

2.1. Materials

Dulbecco's modified Eagle's media (DMEM), antibiotic-antimycotic mix, insulin, rosiglitazone, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Dichloro-dihydro-fluorescein diacetate (DCFH-DA), Naringin and Tertiary butyl hydrogen peroxide (TBHP) were purchased from Sigma—Aldrich Chemicals (St Louis, MO, USA); Monoclonal anti-GLUT4 antibody, secondary anti-mouse immunoglobulin (IgG; Fab specific)—fluorescein isothiocyanate (FITC) antibody produced in goat were purchased from Santa Cruz Biotechnology, USA; Foetal bovine serum (FBS) was purchased from Gibco-BRL (Auckland, NZ); Horse serum was purchased from PAN Biotech (Aidenbach, Germany); 2-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino-2-deoxy-D-glucose (2-NBDG) was purchased from Molecular Probe (Invitrogen Life Technologies, Carlsbad, CA, USA); BCA protein assay kit was procured from Pierce Biotechnology, Rockford, USA; All other chemicals used were of standard analytical grade.

2.2. Cell culture and treatment

L6 myoblast cell line originally isolated from primary cultures of rat thigh was obtained from National Centre for Cell Sciences (NCCS), Pune, India. Differentiated L6 myoblast cell line is the best characterized cellular model of skeletal muscle origin to study glucose uptake and GLUT4 translocation. L6 myoblasts were maintained in DMEM supplemented with 10% FBS, 10% antibiotic-antimycotic mix at 37°C under 5% CO₂ atmosphere. Cells were grown at a density of 1x10⁴ cells/well on 96-well black plates (BD Biosciences, Franklin Lakes, NJ) and 12-well plates (Costar, USA) for the immunofluorescent staining study and glucose uptake assay, respectively.

2.3. Cell viability

Viability of L6 myoblast was measured by means of MTT assay. Cytotoxicity of TBHP and Naringin were standardized based on both concentrations as well as period of incubation. Briefly, cells after incubation with TBHP (1, 10 & 100 μM) and Naringin (1, 10 & 100 μM) were washed and MTT (0.5 g/L) was added to each well for the estimation of mitochondrial dehydrogenase activity as described previously by Mosmann et al [20]. After 4 h incubation, 10% SDS in DMSO was added to each well and the absorbance at 570 nm of solubilized MTT formazan products were measured after 45 min using a microplate reader (BIOTEK-USA). Results were expressed as a percentage of cytotoxicity.

2.4. Measurement of Intracellular reactive oxygen species

Intracellular ROS was estimated by using DCFH-DA following method of Cathcart et al. [21]. Cells were induced with different concentrations of TBHP (1, 10 & 100 μM) to generate oxidative stress, Pretreatment of Naringin was carried for 3 and 24 h respectively. After washing with phosphate buffer saline (PBS, pH-7.4) cells were treated with DCFH-DA (20 μM) for 20 min. The cells were imaged after suspending in PBS using fluorescent microscope (Pathway 855, BD Bioscience, USA) equipped with filters in the FITC range (Excitation, 490 nm; and Emission, 525 nm). The fluorescent intensity was analyzed by BD Image Data Explorer software.

2.5. Lipid peroxidation

Lipid peroxidation was analyzed in L6 myoblast by thiobarbituric acid method [22]. After pretreatment, the cells were lysed and centrifuged at 13,000g for 2 min. Thiobarbituric acid (1ml of 0.67%) dissolved in 5% trichloroacetic acid (TCA) was added to the supernatant. The mixture was heated at 100°C for 15 min. The absorbance was read at 532nm after cooling. Lipid peroxidation was expressed as nanomoles of malondialdehyde (MDA) per million cells, using the MDA extinction coefficient of 156 $\text{mM}^{-1}\text{cm}^{-1}$.

2.6. Determination of Intracellular glutathione concentration

Hissin and Hilf method was followed to determine reduced GSH levels in the cells [23]. Briefly, cells were lysed with metaphosphoric acid and the supernatant thus obtained was mixed with 100 μl ophthaldehyde along with 1ml of phosphate buffer saline. After 15 min, fluorescence was determined at excitation wavelength of 360 nm and emission wavelength of 460 nm. Results were expressed as nanomoles per mg of protein.

2.7. *In vitro* antiglycation assay

To determine the effect on *in vitro* glycation of protein, 500 μl of albumin (1 mg/ml) was incubated with 400 μl of glucose (500 mM) in the presence of 100 μl of Naringin at 1, 10 & 100 μM , respectively. The reaction was allowed to proceed at 60°C for 24 h and 10 μl of 100% TCA was added to stop the reaction. The mixture was centrifuged at 10000 g. Precipitate obtained was dissolved in 500 μl alkaline phosphate-buffered saline (PBS) (pH 10), and the fluorescence was measured at 370 nm (excitation) and 440 nm (emission) [24]. Ascorbic acid serves as a positive control.

2.8. Fluorescence analysis of 2-NBDG uptake by flow cytometry

Glucose uptake by active concentration of Naringin was confirmed by Flow cytometry analysis. Briefly following pretreatment culture medium was removed from each well and replaced with

fresh culture medium in the absence or presence of 10 mM fluorescent 2-NBDG and incubated for 30 min. The cells were then washed twice with cold PBS, trypsinized, resuspended in ice-cold PBS and subjected to flow cytometry. Samples were analyzed using BD FACS Aria II (BD Biosciences) at FITC range (excitation 490 nm, emission 525 nm band pass filter). The mean fluorescence intensity of different groups were analyzed by BD FACS Diva software and corrected for autofluorescence from unlabeled cells.

2.9. Immunofluorescence staining

To investigate the molecular mechanism of the induction of glucose uptake GLUT4 upregulation was monitored by Laser based confocal imaging. After pretreatment with the Naringin (100 μ M) and TBHP, cells were washed with PBS and fixed for 5 min with 4% formaldehyde and permeabilized with triton-X for 10 min. Cells were blocked with 5% BSA for 1 h followed by incubation with monoclonal GLUT4 antibody solution (1:200 dilution in 1.5% BSA in PBS) at 4°C overnight. And then incubated with FITC-conjugated goat anti-mouse IgG secondary antibody (1:500 dilution, 1.5% BSA in PBS) for 1 h. The cells were also counter stained with nuclear stain (DAPI- (4',6-diamidino-2-phenylindole)). Images were acquired using Laser Scanning Confocal microscope (Nikon A1R, Nikon Instruments, Melville, USA) equipped with filters in the FITC range (i.e. excitation, 490 nm; and emission, 525 nm). Images were analyzed by NIS ELEMENTS software.

2.10. Statistical analysis

Results were expressed as means and standard deviations of the control and treated cells from triplicate measurements ($n = 3$) of three different experiments. Data were subjected to one-way ANOVA, the significance of various groups were calculated by Duncan's multiple range test using SPSS for Windows, standard version 16 (SPSS, Inc.) and significance was accepted at $P \leq 0.05$.

Results

3.1. Cell viability

The cytotoxicity of TBHP was standardized based on concentration as well as period of incubation. TBHP and Naringin at 100 μ M was found to be less than 20 (Fig 1A) and 15% toxic (Fig 1B) for a period of 3 and 24 h respectively. These concentrations were taken for further studies.

3.2. Determination of intracellular ROS

To investigate the effect of Naringin on oxidative stress associated with diabetes mellitus we induced stress in L6 skeletal muscle cells by using TBHP. Induction of free radicals with TBHP at three different concentrations (1, 10 & 100 μ M) for 3 h revealed that cells generated significant levels of intracellular reactive oxygen species as compared to control (Fig 2A), ($P \leq 0.05$). TBHP at 100 μ M (Fig 2A (iv)) showed a significant increase in intracellular ROS and this concentration was used to induce stress condition in further studies. Pretreatment of Naringin for 3 & 24 h at different concentrations (1 μ M, 10 μ M & 100 μ M), dose dependently reduced ROS concentration as shown in Fig 2B & 2C ($P \leq 0.05$). The fluorescence intensity of the images was analyzed by BD Image Data Explorer software and has been illustrated in Fig 2A(v), 2B(vi) & 2C(vi).

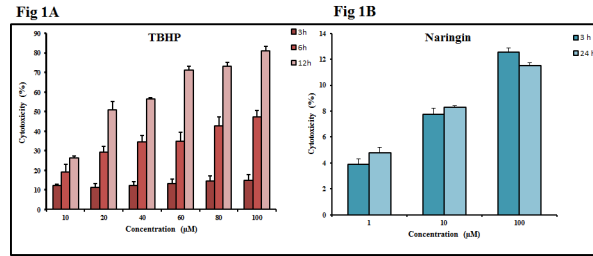


Fig 1. Cytotoxicity in cultured L6 myoblast. (A) Effect of TBHP on cell viability was evaluated based on concentration as well as period of incubation. (B) Cytotoxicity of Naringin was determined after 24 h preincubation in L6 myoblast. Each value represents mean \pm SD (standard deviation) from triplicate measurements ($n = 3$) of three different experiments.

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3.3. Influence in lipid peroxidation

There was a significant increase in malonaldehyde concentration in L6 myoblast on induction of oxidative stress (0.513 nmol) than that of untreated control (0.253 nmol) ($P \leq 0.05$). Pretreatment with Naringin even at 1 μ M restricted the production of malondialdehyde to 0.39 nmol and Naringin at 10 & 100 μ M showed a decrease to 0.36 & 0.29 nmol, respectively ($P \leq 0.05$) (Table 1).

3.4. Role in GSH metabolism

GSH levels were monitored after 3 and 24 h of Naringin (1, 10, and 100 μ M) pretreatment to investigate the effect of Naringin in natural antioxidant defense system of L6 cells. Oxidative stress induced by TBHP reduced GSH level by 34% compared to control ($P \leq 0.05$). Naringin pretreatment retrieved the GSH level to that of control as shown in Table 1.

3.5. Effect on Non-enzymatic glycation

Non-enzymatic glycosylation between reducing sugar and protein results in the formation of advanced glycation end products (AGEs), which is involved in diabetic complications. Thus, compounds that inhibit the formation of AGEs are believed to have therapeutic potentials in patients with diabetes. Naringin showed a marked decline in *in vitro* glycation of proteins in a dose dependent manner (Fig 3). Naringin at 100 μ M showed an inhibition of 40%. Ascorbic acid was used as a positive control ($IC_{50} - 30 \mu$ M).

3.6. Flow cytometric analysis of glucose uptake

2-NBDG uptake in L6 myotubes was analyzed by flow cytometry by detecting the fluorescence within the cells. There was no significant effect on 2-NBDG uptake in the cell induced with TBHP (8.7%) compared to control (8%) (Fig 4). At 24 h pretreatment of Naringin (10 μ M and 100 μ M), the glucose uptake in L6 myotubes remarkably increased to 27.5% and 35.9%, which was much higher than that of positive control, Rosiglitazone (30.4%, $P \geq 0.05$).

3.7. Upregulation of GLUT4 in L6 myotubes

Immunofluorescence staining revealed an upregulation of GLUT4 transporter protein on pretreatment of Naringin (100 μ M) which was comparable to that of positive control as shown in Fig 5 ($P \geq 0.05$). The results obtained by quantifying immunologically labeled GLUT4 at the surface of intact cells correlates with that of glucose uptake, suggesting that the induction of GLUT4

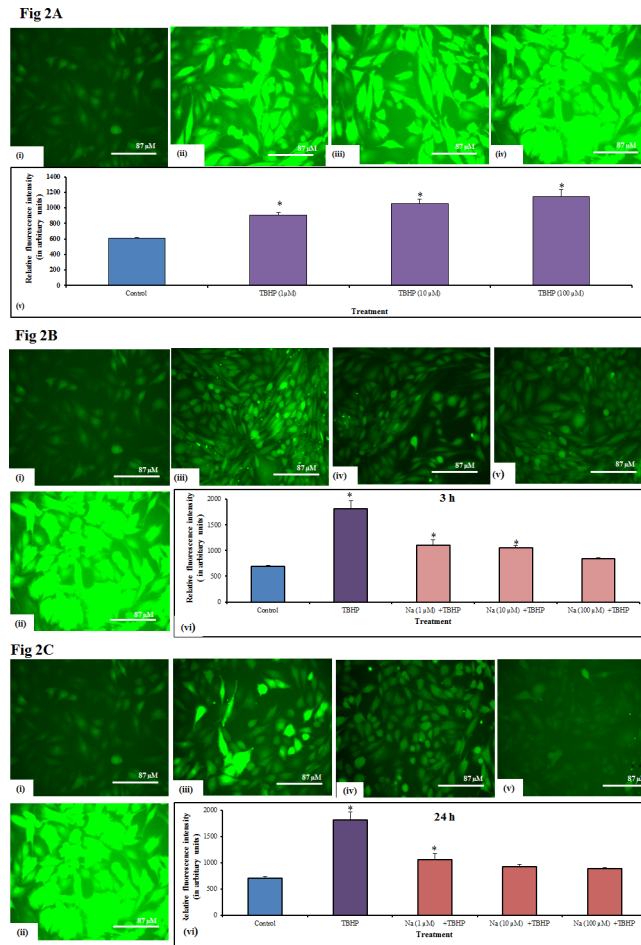


Fig 2. Intracellular ROS production and Fluorescence intensity analysis in L6 myoblast. (A) Fluorescence images (20 X magnifications) of untreated cell (i); Figures (ii), (iii) & (iv) represents cells induced with TBHP at 1, 10 & 100 μM; (v) Represents the relative fluorescence intensity analysis by BD Image Data Explorer software. Significance test between various groups was determined by using one way ANOVA followed by Duncan's multiple range test. * $P \leq 0.05$ versus control. (B) Figure (i), (ii), (iii), (iv) & (v) represents fluorescence images of untreated cells, cells induced with TBHP (100 μM) and cells pretreated with Naringin (1, 10 & 100 μM) for 3h respectively; (vi) Represents the relative fluorescence intensity analysis by BD Image Data Explorer software. Significance test between various groups was determined by using one way ANOVA followed by Duncan's multiple range test. * $P \leq 0.05$ versus control; # $P \leq 0.05$ versus TBHP. (C) Figure (i), (ii), (iii), (iv) & (v) represents fluorescence images of untreated cells, cells induced with TBHP (100 μM) and cells pretreated with Naringin (1, 10 & 100 μM) for 24h respectively; (vi) Represents the relative fluorescence intensity analysis by BD Image Data Explorer software. Scale bar corresponds to 87 μM. TBHP: Tertiary butyl hydrogen peroxide; Na1+TBHP, Na2+TBHP & Na3+TBHP represents relative fluorescence intensity analysis of cells pretreated with Naringin (1, 10 & 100 μM) followed by induction of TBHP; Each value represents mean \pm SD (standard deviation) from triplicate measurements (n = 3) of three different experiments. Significance test between various groups was determined by using one way ANOVA followed by Duncan's multiple range test. * $P \leq 0.05$ versus control; # $P \leq 0.05$ versus TBHP.

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by pretreatment of Naringin is likely responsible for enhanced glucose uptake exhibited by L6 myotubes. TBHP exposure to cells did not induce any changes in GLUT4 translocation.

Discussion

Over the past decade, investigators had shown tremendous interest in studying the role of oxidative stress markers in the genesis of diabetes and its associated complications. Defective

Table 1. GSH, MDA levels in L6 myoblast after treatment in cultured L6 myoblast. The levels of GSH and MDA were assayed after treatment with Naringin followed by induction of oxidative stress in L6 myoblast. Each value represents mean ± SD (standard deviation) from triplicate measurements (n = 3) and the significance accepted at P ≤ 0.05.

Treatment	Dose (µM)	MDA/10 ⁴ cells (nmol)		GSH (nmol/mg of protein)	
		3h		3h	24 h
Untreated control	-	0.253±0.099		23.91± 2.13	
TBHP	100	0.513±0.019*		15.78 ±1.75*	
Naringin	1	0.39±0.088 [#]		18.17±0.99 [#]	20.10±1.07 [#]
	10	0.361±0.078 [#]		20.38±1.01 [#]	25.20±0.97 [#]
	100	0.29±0.055 [#]		27.31±1.20 [#]	27.70±0.49 [#]

* P ≤ 0.05 versus Control

[#] P ≤ 0.05 versus TBHP

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antioxidant system has been reported in diabetic patients compared to healthy subjects [25]. GSH, an abundant and ubiquitous antioxidant, function mainly as an efficient intracellular reductant, protecting cells from damage caused by free radical, drugs and radiation. Decreased GSH in TBHP treatment may be due to its increased utilization in protecting cells. In the present study, GSH was retrieved to normal levels in L6 myoblast on pretreatment with Naringin. GSH is an important substrate for glutathione peroxidase (GPx) and glutathione-S-transferase, which is an inevitable part of our antioxidant system. Naringin treatment is reported to upregulate the gene expression of GPx in high cholesterol fed rat [26].

Defects in the antioxidant defense system are in close association with induction of lipid peroxidation [27]. We have observed increased level of malondialdehyde, a byproduct of lipid peroxidation on induction of oxidative stress with TBHP. Chronic pretreatment of Naringin significantly decreased thiobarbituric acid reactive substances levels, and our results are in agreement with Rajadurai et al. [28] who reported a decrease in mitochondrial lipid peroxidation in Wistar rats on Naringin pretreatment. Naringin has also been reported to improve catalase and superoxide dismutase activities in the heart of isoproterenol induced rats [29].

AGE's and their derivatives are reported to contribute significantly to the pathogenesis of diabetes and elicit oxidative stress that elevates diabetic complications. In our study, a significant decrease in glycation of bovine serum albumin was found on coinubation of Naringin at

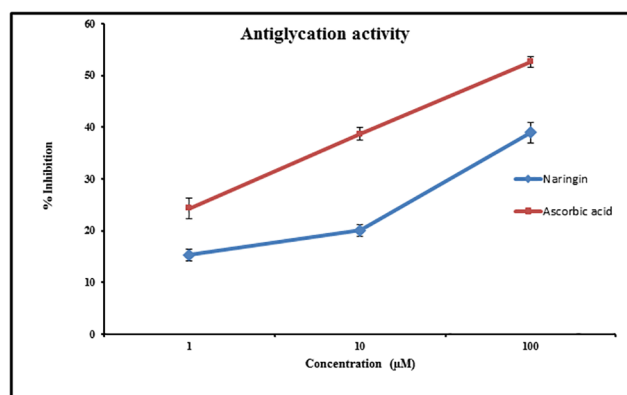


Fig 3. Antiglycation activity of Naringin. Antiglycation activity of Naringin at three different concentrations (1, 10 & 100 µM). Ascorbic acid was used as standard. Each value represents mean ± SD (standard deviation) from triplicate measurements (n = 3) of three different experiments.

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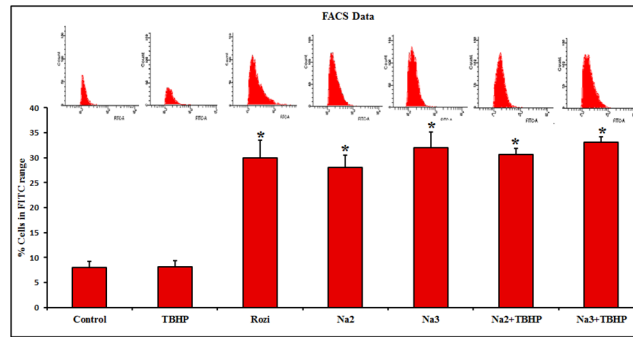


Fig 4. Fluorescence analysis of 2-NBDG uptake by flow cytometry. FACS analysis of 2-NBDG uptake in differentiated L6 cells by plotting cell count against FITC revealed that 8%, 8.1% and 30% of cells uptake 2-NBDG in control, TBHP and Rosiglitazone treated cells respectively whereas 30.6%, 33.1%, 28%, 32% of cells uptake 2-NBDG, pretreated with two different concentrations (10 and 100 μ M) of Naringin along with/ without TBHP respectively. Each value represents mean \pm SD (standard deviation) from triplicate measurements (n = 3) of three different experiments. Significance test between various groups was determined by using one way ANOVA followed by Duncan's multiple range test. * $P \leq 0.05$ versus control.

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100 μ M. A number of AGE inhibitors, including aminoguanidine, improved diabetic complications in both animal models and clinical trials but are reported to possess numerous side effects [30]. Thus, Naringin may be considered as a promising candidate to reduce AGE's formation owing to its lesser side effects. Though anti glycation activity of many of the flavonoids has been extensively studied, there are only a few reports on the anti glycation property of Naringin.

Several clinical trials have demonstrated that treatment with antioxidants, such as, vitamin E, vitamin C or glutathione, improved insulin sensitivity in insulin resistant individuals [31, 32]. In the present study, we observed a substantial increase in glucose uptake in L6 myotubes on pretreatment of Naringin (100 μ M) which was much higher than that of the antidiabetic drug, rosiglitazone. In our previous study, we have made a comparative evaluation of

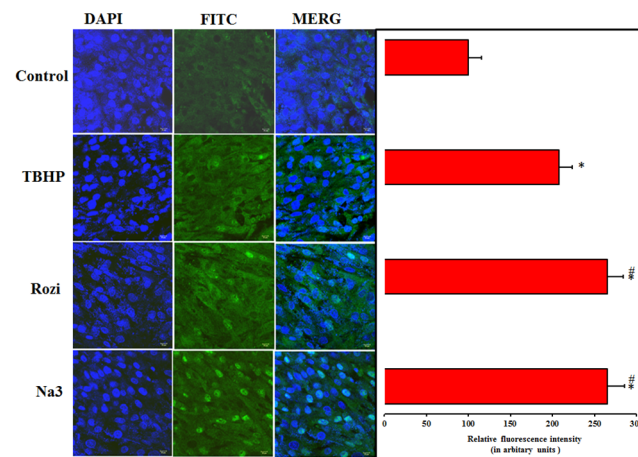


Fig 5. GLUT4 upregulation on Naringin pretreatment. Immunofluorescence assay visualized upregulation of GLUT4 in differentiated L6 myoblast. High resolution confocal images (40X) of Untreated L6 myotubes, L6 myotubes treated with TBHP, Rosiglitazone (100 nM) and Naringin (100 μ M, 24 h). Scale bar corresponds to 10 μ M. Each value represents mean \pm SD (standard deviation) from triplicate measurements (n = 3) of three different experiments. Significance test between various groups were determined by using one way ANOVA followed by Duncan's multiple range test. * $P \leq 0.05$ versus control; ## $P \leq 0.05$ versus TBHP.

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antidiabetic potential of Quercetin and Rutin, which pointed to the role of chemical modification on the biological activities of the flavonoids [33]. Inducing glucose uptake in muscle cells can have a remarkable effect on plasma glucose level owing to its large proportion in human body. Although our primary focus is on skeletal muscle, it is possible that Naringin may increase glucose uptake in the liver and fat tissue in addition to the effect in muscles. Mahmoud et al. [34] reported that pretreatment of hesperidin and Naringin decreased the levels of glucose, glycosylated hemoglobin, MDA, nitric oxide, TNF- α and IL-6 in streptozotocin induced rats. Naringin is also reported to modulate hepatic glycolysis and gluconeogenesis [35]. In addition, we found that Naringin pretreatment induced translocation of GLUT4 from intracellular compartments to plasma membrane to the same extent than that of rosiglitazone pointing to the molecular end mechanism utilized by Naringin in inducing glucose uptake.

Conclusion

Strategies to control oxidative stress have become a relevant pharmacotherapy in the treatment of micro and macrovascular diabetic complications. Antioxidant like naringin, acting on different molecular targets of diabetes appears to be promising in the management of both diabetes and its associated complications. Therapeutic potential of natural antioxidants needs to be explored further to reveal their effect on molecular signaling.

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Author Contributions

Conceived and designed the experiments: RD PJ TRSK PN. Performed the experiments: RD PJ KBA HPS. Analyzed the data: RD VMN PJ. Contributed reagents/materials/analysis tools: HPS VMN TRSK. Wrote the paper: RD PJ PN.

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