Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v29i01.3303

EFFICACY OF NARINGIN IN PROTECTING CARDIAC H9C2 CELLS FROM HYPERGLYCEMIC DAMAGE: POTENTIAL IMPLICATIONS FOR DIABETIC CARDIOMYOPATHY TREATMENT

Amer Mahmood[1]*, Randa AL-Ibrahim[1], Mona Elsafadi[1], Manikandan Muthurangan[1], Abdul-Aziz Siyal[1], Randa Alfotawi[2]

[1]Stem Cell Unit, Anatomy Department, Medical School, King Saud University, Riyadh, Saudi Arabia, email: melsafadi@ksu.edu.sa, Mrangan@ksu.edu.sa, asiyal@ksu.edu.sa
[2]Oral and Maxillofacial Department, King Saud University, Riyadh, Saudi Arabia

***Corresponding Author:** Amer Mahmood
ammahmood@ksu.edu.sa

Abstract

Diabetes mellitus (DM) represents a pressing public health challenge globally, with profound implications in both developed and developing nations. It stands as one of the most pervasive and severe chronic conditions worldwide. This study delves into the potential therapeutic effects of naringin on H9c2 cardiac cells subjected to elevated glucose levels, specifically exploring its viability as a treatment for diabetic cardiomyopathy.

Methods: For our experiment, we prepared a final concentration of 80.0 µM of NAR in distilled water, subsequently stored at 4.0°C. H9c2 cardiac cells, derived from embryonic rat ventricles, were cultured in DMEM supplemented with FBS, D-Glucose. In this study we simulated Diabetic Cardiomyopathy by exposing the cells to high D-Glucose concentrations. Viability was assessed using the AlamarBlue assay, while ROS production was detected using the DCFH–DA dye. RNA from treated and control cells was isolated to test different genes.

Results and discussion: In summary, pre-treatment of H9c2 cardiomyocytes with 80.0 µM concentrations of both Naringenin and Naringin, prior to high glucose exposure, proved most effective, especially concerning the upregulation of the Bcl-2 anti-apoptotic gene. The present study explored the adverse impacts of high glucose (HG) on H9c2 cardiomyocyte cells, evidenced by symptoms like cytotoxicity, apoptosis, oxidative stress, and mitochondrial malfunctions, further underlined by a reduced cell viability. We discovered a pronounced linkage between the surge in reactive oxygen species (ROS) production and HG-driven cardiomyocyte damage.

Conclusion: Naringenin (NAR) at 80.0 µM concentration, when pre-treated on H9c2 cardiomyocytes prior to high glucose exposure, effectively upregulates the Bcl-2 anti-apoptotic gene, offering protection against hyperglycemia-triggered cardiac damages by reducing ROS activity and cell apoptosis. The study reaffirms previous findings linking elevated HG levels to cardiomyocyte damage, emphasizing that an 80.0 µM NAR treatment for 2.0 hours is the most potent countermeasure against diabetic cardiomyopathy.

Introduction

Diabetes mellitus (DM) is one of the significant public health troubles in both developed and developing countries and one of the most essential chronic severe diseases in the world. Diabetes

mellitus is a major global health concern, the International Diabetes Federation (IDF) estimated that DM would be one of the most severe causes of death by 2040, and the number of adults who suffered from DM now about 424.000.000 and will rise highly to 641.000.000 by 2040 (1). Three million eight hundred fifty-two thousand cases of diabetes in Saudi Arabia alone and this number is projected to increase to 7.691.000 by 2045 (2). Diabetes mellitus describes a group of metabolic disorders characterized by increased blood glucose levels. Diabetes mellitus and its complications have contributed tremendously to the burden of mortality and health cost worldwide (3). Persistently high blood glucose levels causes damage, and failure of various organ systems cause generalized vascular damage affecting the heart, eyes, kidneys, nerves and resulting in various complications (4). Diabetes mellitus affects the heart specifically through different mechanisms, including microvascular impairment, metabolic disturbance, subcellular component abnormalities, cardiac autonomic dysfunction, and a maladaptive immune response (5). Also, diabetes mellitus can cause functional and structural changes in the myocardium without coronary artery disease, valvular heart disease, and other cardiovascular risk factors, such as hypertension and dyslipidemia a disorder known as diabetic cardiomyopathy (DCM) (6). Heart failure and related morbidity and mortality are increasing at an alarming rate, in large part, because of increases in aging, obesity, and diabetes mellitus. The clinical outcomes associated with heart failure are considerably worse for patients with diabetes mellitus than for those without diabetes mellitus (7). Diabetic complications remain the leading cause of morbidity and mortality in diabetic patients, with cardiovascular disease being the principal reason for morbidity and mortality among diabetic patients, including atherosclerosis, myocardial infarction, and cardiomyopathy (8). Diabetic cardiomyopathy is one of the main complications among DM patients. Diabetic cardiomyopathy characterized by structural and functional alterations in the myocardium, such as cardiac hypertrophy, oxidative stress, inflammation, apoptosis, and myocardial interstitial fibrosis. Persistent hyperglycemia causes many molecular and metabolic changes in cardiomyocytes (9). Increased glucose metabolism due to hyperglycemia increases oxidative stress through mitochondrial reactive oxygen species (ROS) production. Oxidative stress leads to reduced myocardial contractility and eventually induces myocardial fibrosis (10). In addition, oxidative stress can accelerate cardiomyocytes apoptosis and cellular DNA damage. Moreover, mitochondrial dysfunction and increased oxidative stress play a pivotal role in the development of diabetic cardiomyopathy and associated heart failure by increased glucose metabolism due to increases of hyperglycemia that lead to increase of oxidative stress via the production of ROS from the mitochondria (11).

The basic aim of the study is to find the effects of naringin on cardiac cells H9c2 Treated with high concentration of glucose as a possible treatment for diabetic cardiomyopathy disease**.**

MATERIALS AND METHODS

The practical part of this research was executed at Stem Cells Unit, Department of Anatomy, Faculty of Medicine, King Saud University, and Electron Microscope Unit, King Faisal Specialist Hospital from March /2019 to December /2019.

Preparation of Stock Solutions

Dissolve 11.6 g NAR in 100.0 ml dH_2O to give 200.0 mM as stock solution and stored at 4.0 ^oC. Then to make 80.0 µM final concentration, we dissolved 4.0 µl from a stock solution in 1000.0 µl DMEM medium.

Culture Media

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10.0% Fetal Bovine Serum (FBS), 4.5 g/L D-Glucose, l10.0 mg/L Sodium Pyruvate, 1.0% Penicillin and Streptomycin (10.000 units of penicillin and streptomycin/mL) and 1.0% Nonessential Amino Acids (NEAA) (100X). This medium used for cell growth and cell proliferation. Prepared culture medium stored at 4.0^oC.

Cell Line

H9c2 cell line used in this study is a clonal myogenic cell derived from embryonic rat ventricle that can assist as an alternative source for cardiac cells *in vitro*. These cells were obtained from Dr. Hafedh Dakheel (Obesity chair, Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia).

Experimental design

This experiment was designed to investigate the effect of a high concentration of D-Glucose (35.0 mM), as a way to create a Diabetic Cardiomyopathy situation on the normal H9c2 cardiac cells. Cells were cultured in medium supplemented with a high concentration of glucose (Positive control) or without (Negative control), according to Chen *et al.* (2013) for other 24.0 hrs. After the culture period and cells were reached to approximately 80.0 % confluence, the cells were trypsinized by adding 0.05 % trypsin and the cell suspensions were used to count the cells in the flask. The cells were counted using the Neubauer hemocytometer-counting chamber (Paul Marienfeld Gmbh & CO.KG.) by placing 10.0 µl of cell suspension in the upper chambers and lower cover of the hemocytometer slide, and then the cells were counted manually using inverted microscope provided with image software under 10X magnification. Number of cells was calculated as the following equation from (Dilution Calculator) (Ellison, 2018) :-

$C1 \times V1 = C2 \times V2$

C1: Total cell number (cell/ml).

C2: The total number of cells needed ((cell/ml)/well). V1: Unknown volume.

V2: Volume of media.

Measurement of Cell Viability Using AlamarBlue Assay

Cells were plated into 96-well plates that used to test cell viability and proliferation (Figure 6). Cell viability was measured using AlamarBlue assay reagent according to the manufacturer's recommendations, as described by Rampersad (2012). Briefly, 0.012 X 10^6 cell/well was seeded and cultured in 96 well plates contained 100.0 µl DMEM medium/well and exposed to NAR at different concentrations and times as shown above in the experimental design. While, cells were exposed to NG for 2.0 hrs. only at 80.0 µM concentration. Then, all cells treated with both NAR and NG were exposed to high glucose (HG) concentration (35.0 mM) for 24.0 hrs. After treatment with HG, 10.0 µl (10.0 % of medium volume) of AlamarBlue reagent was added to each well, and the plates were incubated in the dark at 37.0°C for 2.0 hrs. In the end, the plates were taken to read using a fluorescent microscope (Ex 530 nm/Em 590 nm) and Bio-Synergy II microplate reader (BioTek Inc., Winooski, VT, U.S.A.).

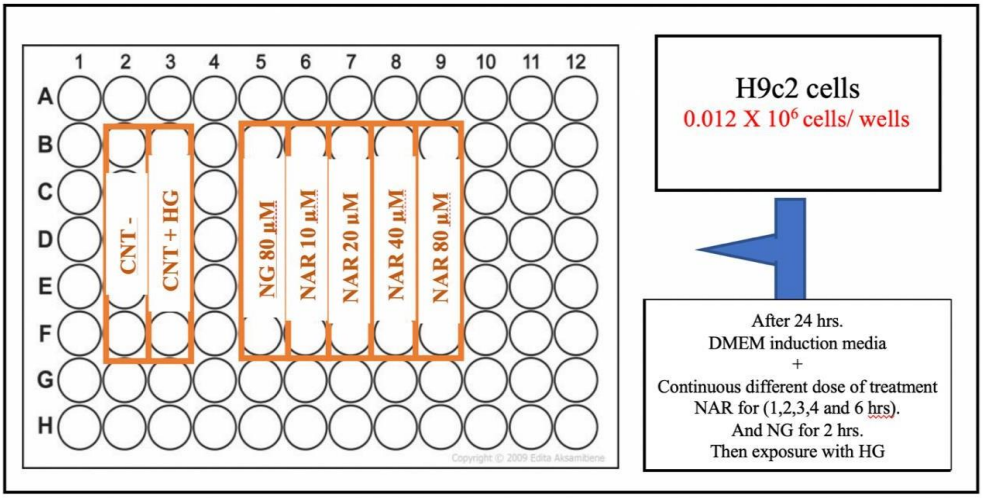

Figure 1. Plate Strategy for measurement of cell viability and proliferation.

Detection of Reactive Oxygen Species (ROS)

Reactive Oxygen Species production was detected using the Dichlorofluorescin hydrochloride Diacetate (DCFH–DA) dye, which is converted to Dichlorofluorescin hydrochloride (DCFH) by intracellular esterase. Inside the cells, DCFH is converted to the highly fluorescent 2',7'–dichlorofluorescein (DCF) due to oxidation by ROS.

RNA Isolation

According to the protocol and precautions of the total RNA Purification Kit (table 3), RNA was isolated from H9c2 cardiac cells resulted from all treatments and control groups after culture. Extraction to breakdown the cells and allow the cells components to be freely released (DNA, RNA, and protein). RNases are very stable and robust enzymes that degrade RNA. Therefore, the first step when preparing to work with RNA is to create an RNase-free environment. All centrifugations were carried out in benchtop micro- centrifuge at room temperature and speed was 12,000 RPM/min.

Statistical Analysis

Statistical analysis was performed using the SPSS software program. All data were analyzed as a completely randomized design using an independent sample t-test to compare data of negative and positive control and One-Way Analysis of Variance (ANOVA) for other data of treatments, followed by Duncan's test to compare the significance between means. Comparisons between means considered significant at $p \leq 0.05$. Results were presented as a mean ± standard error of mean (SEM).

Funding and Acknowledgement

This work was funded by the National Plan for Science, Technology and Innovation (**MAARIFAH**), King Abdul-Aziz City for Science and Technology, Kingdom of Saudi Arabia, grant Number **14-MED715-02**.

RESULTS

Effect of High Concentration of Glucose (35.0 mM) on Cardiomyocytes H9c2 Cells

Cell Viability

Cell viability results showed a highly significant difference between the negative control group (CNT) and the positive control group (HG). The negative control showed high cell viability (12226.00 □ 110.27) than HG group that showed reduced cell viability (4904.00 □ 122.87) as shown in table 1.

Table 1. Effect of high concentration of glucose (35.0 mM) on the viability of H9c2 Cardiomyocytes

Treatments / Trait	Negative Control (CNT)	Positive Control High Glucose (HG)
Cell viability	a 12226.00 □ 110.27	b 490400.00 □ 122.87

Values in the same row with different superscripts (a, b) differ significantly at $P \leq 0.05$.

Cell Morphology

Cardiomyocytes (H9c2) exposed to high glucose have changed morphology when compared with a negative control. In the HG group, there were more round and flatter cells than in the negative control group. Moreover, we observed that the nuclei got compact and the chromatin appeared shrunk.

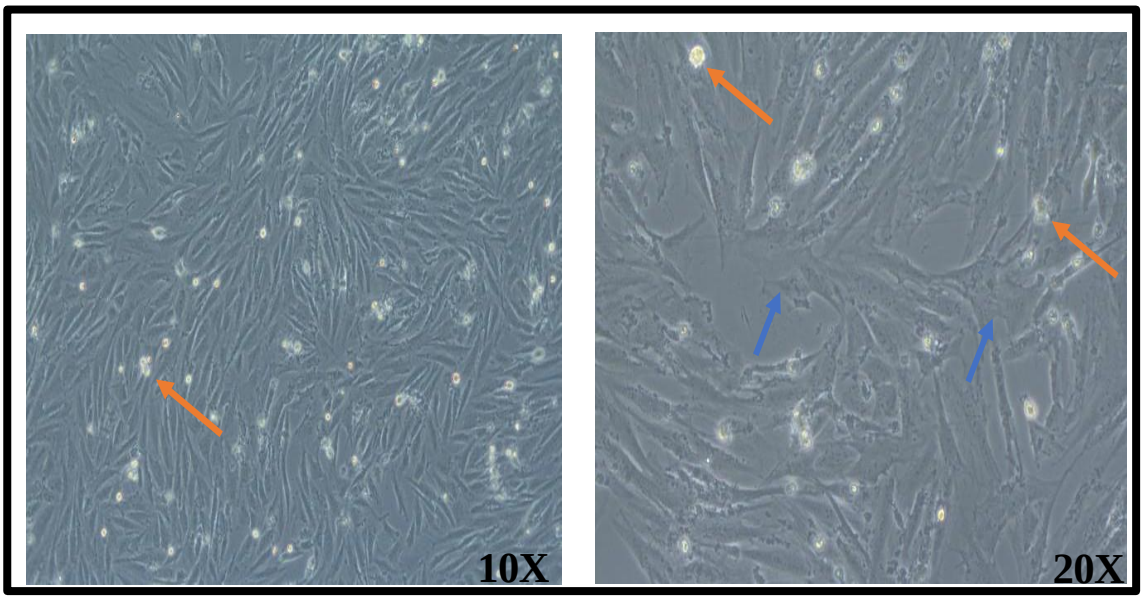

Figure 2. Morphology of H9c2 rat cardiomyocytes cells exposed to a high concentration of glucose as a positive control showed round cells (Orange arrow) and flat cells (Blue arrow) at different magnifications (10 and 20 X).

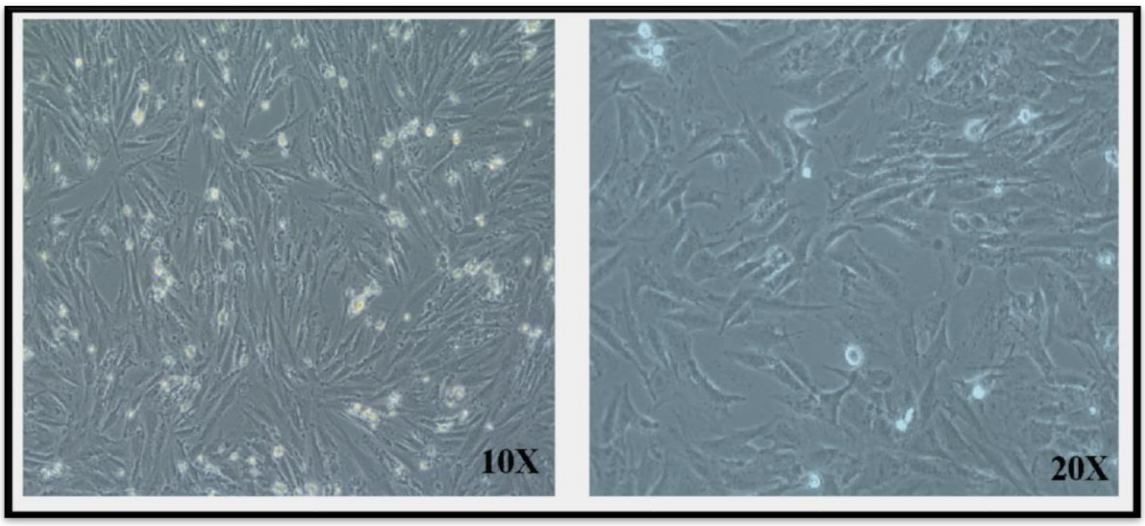

Figure 3. Normal morphology of H9c2 rat cardiomyocytes cells in negative control group at different magnification (10 and 20 X).

Reactive Oxygen Species (ROS)

Positive control group showed a significant increase mean of ROS (6.93 □ 0.31) when compared with a negative control group (1.21 □ 0.10), which was also observed in figure 13. So, the positive control group was the worse than the negative control group.

Table 2. Reactive oxygen species induced by high glucose in H9c2 cardiomyocytes

Trait / Treatments	Time (hrs.)	Reactive Oxygen Species
Negative Control	24.0	b 1.21 □ 0.10
Positive Control High Glucose (HG)		a 6.93 □ 0.31

Mean values in the same column with different superscripts (a, b) differ significantly at P ≤0.05

Live and Dead Cells

Table (2) showed the mean values of live cell ratios in negative control group compared with positive control group. The mean values of live cell ratios was significant increase in positive control (84.93 □ 2.19) than negative control (97.93 □ 0.72). Also, examined with fluorescent microscope using fluorescent dyes revealed more apoptotic cells (dead cells) in positive control than in negative control group. Then, the negative control group was the best than the positive control group.

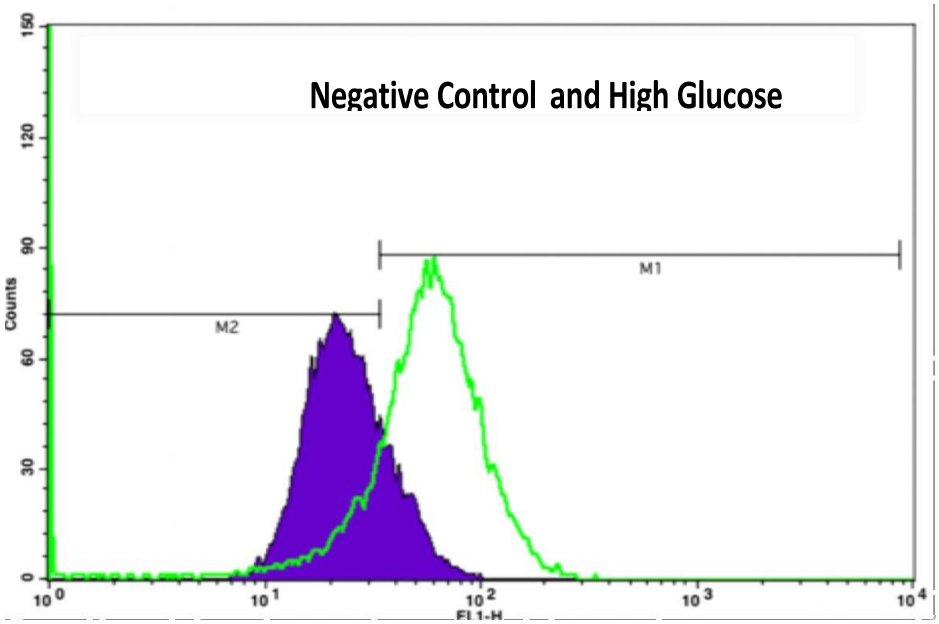

Figure 4. Diagram showed the flow-cytometry of reactive oxygen species in H9c2 rat cardiomyocytes for negative control group (Violet peak) and the high concentration of glucose as a positive control group (Green peak).

Table 3. Effect of high concentration of glucose (35.0 mM) on mean of H9c2 live cell ratio

Trait / Treatments	Time (hrs.)	Live cells ratio
Negative Control	24.0	a 97.93 □ 0.72
Positive Control High Glucose (HG)		b 84.93 □ 2.19

Mean values in the same column with different superscripts (a, b) differ significantly at P ≤0.05

Gene Expression of Cell Regulation Genes (Nkx2.5, cdkn1b, and ccnd1)

The Gene expression of the cell regulation genes (Nkx2.5, cdkn1b, and ccnd1) in cardiomyocytes H9c2 cells showed the downregulate effects concerning with the positive control compared with the negative control for all tested genes. The foregoing indicates that the negative control group is better than the positive control group.

Gene Expression of Anti-Apoptotic Bcl-2 Gene

It showed the comparison between two control groups (negative and positive) for expression of Bcl-2 gene. Results showed decrease of gene expression mean in negative control group (0.66) compared with positive control group (1.00). Therefore, we can say that the negative control group is better than the positive control group regarding the unstudied anti-apoptotic gene (Bcl-2).

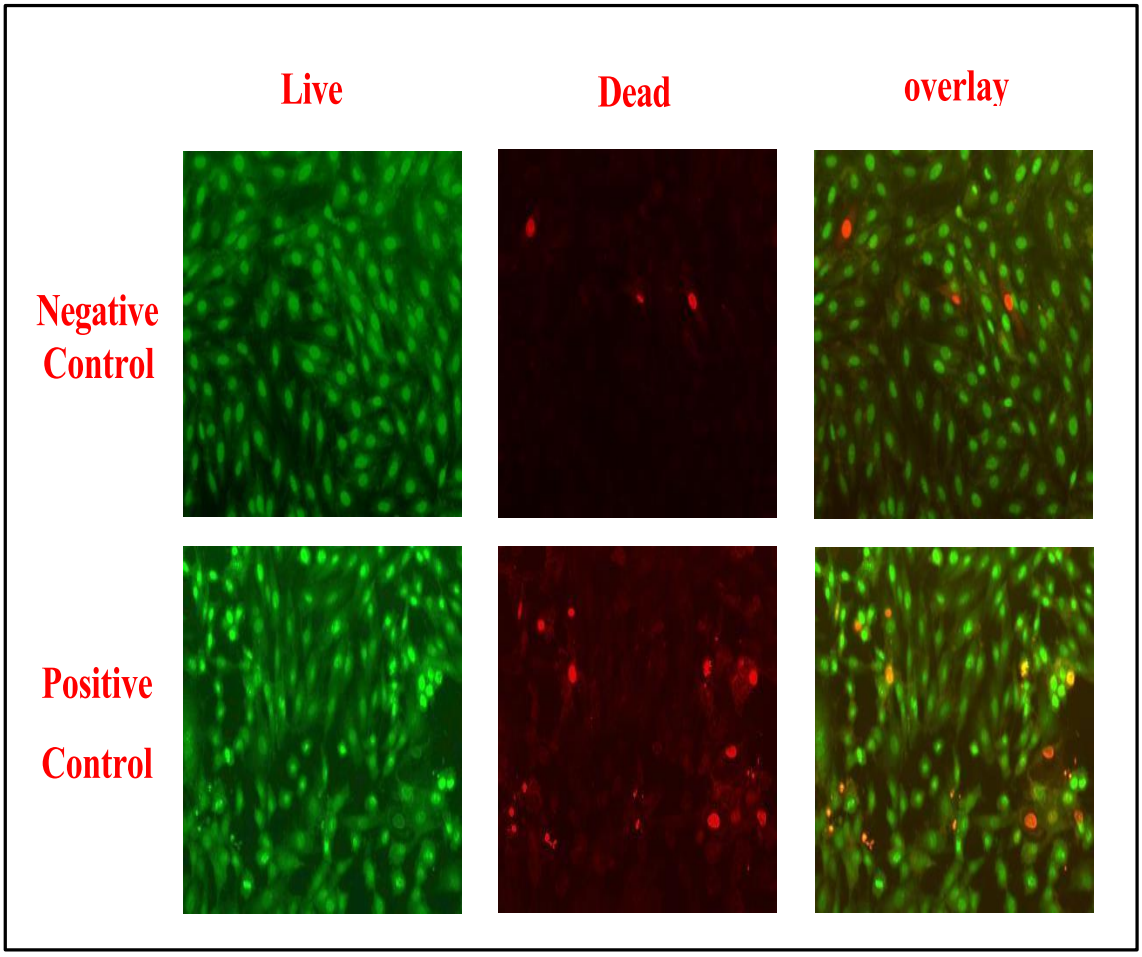

Figure 5. Images of H9c2 rat cardiomyocytes cells stained with fluorescent dyes showed live cells (Green color) and dead cells (Red color).

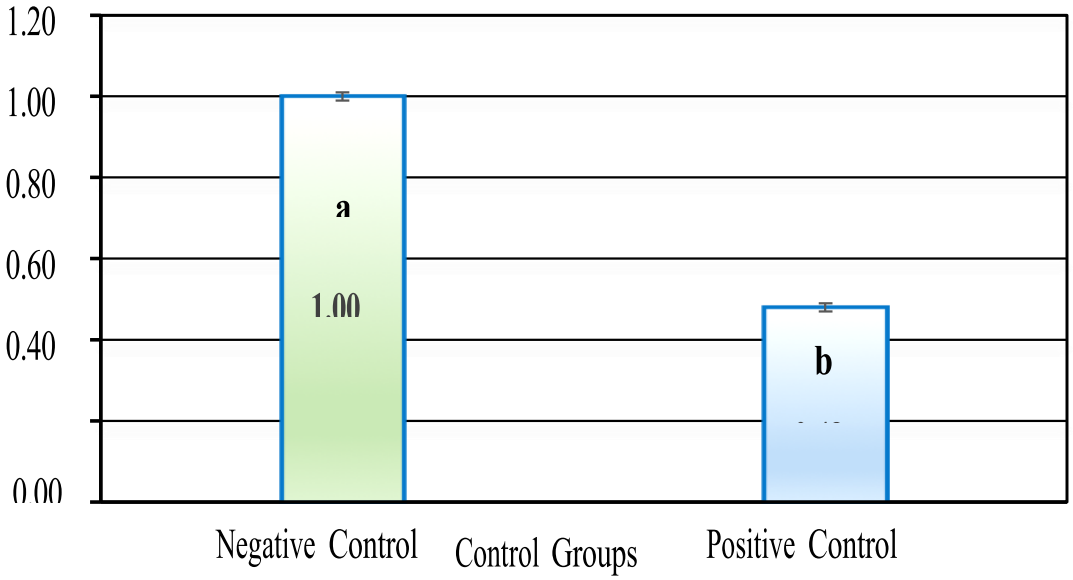

Figure 6. Comparison of gene expression between negative and positive control groups of cell regulation **Nkx2.5 gene**.

DISCUSSION

In the current study, we found that high glucose (HG) induced multiple deteriorative effects on H9c2 cardiomyocyte cells, including cytotoxicity, apoptosis, oxidative stress and mitochondrial abnormalities, and evidenced by a decrease in cell viability (9-11). The over-production of ROS is not

only an index of oxidative stress, but also one of the mechanisms underlying HG- induced cardiomyocyte injury. Wherefore, we provide a new insight concerning with the possible protection of hyperglycemia-induced cardiac cell injuries using NAR by inhibiting the activation of ROS, mitochondrial damage and cell apoptosis (12).

First, we investigated the effect of exposure of high glucose (35.0 mM) on cardiomyocyte cell line, as positive control group. The results showed increased cytotoxicity, and decreased cell viability compared with negative control group (35.0 mM Glucose), these results were consistent with previous studies (13-16). Their results showed that HG levels are the main reason for hypertrophy by increasing the surface area of the heart and decreased cell viability (17).

In addition, we investigated the toxic effect of Naringenin **(**NAR) on untreated H9c2 cells with HG. The results showed clearly that NAR has no toxic effect on H9c2 cells and these results were in agreement with the results of previous studies (18). These studies showed the effect of treatment with NAR on H9c2 cells and they did not find any toxic effect for NAR as well (19). Thereafter, H9c2 cells were treated with different concentrations of NAR at different time points showed previously in experimental design before expose to HG to investigate the protective effects of NAR against HG-induced damage (20). The maximum protective effect of NAR against HG was achieved at 80.0 μM concentration for 3.0 hrs., exposer time, where the results showed a significant increase in cell viability compared with positive control group (21). So, our results proved that the high concentration of NAR is better than lower concentrations, and these results were in agreement with Tang *et al.* (2016), which showed in their study that the effective protection of naringenin substance was at 80.0 μM concentration against the cellular toxicity characteristic caused by Hypoxia and Reoxygenation (H/R) (22-23). In addition, in another study by Zhang *et al.* (2019), they found that the better results were achieved when treated H9c2 with high concentration of NAR substance (10.0 μM) than lower concentrations (0.1, and 1.0 μM) with regard to the treatment of cardiomyopathy. Likewise, the treatment of H9c2 cells with NG substance at 80.0 μM concentration for 2.0 hrs. gave the best results in the treatment of diabetic cardiomyopathy (24-26).

Conclusion

In conclusion, the treatment of cardiomyocytes H9c2 cells with 80.0 μM from Naringenin and Naringin substances before exposed to high glucose concentration were the best treatments with regard to the expression of Bcl-2 anti-apoptotic gene.

References

1. Pozzo, E., Costa, B., Cavallini, C., Testai, L., Martelli, A., Calderone, V., & Martini, C. (2017). The Citrus Flavanone Naringenin Protects Myocardial Cells against Age-Associated Damage. *Oxidative medicine and cellular longevity, 2017*, 9536148. doi: 10.1155/2017/9536148
2. Qin, W., Ren, B., Wang, S., Liang, S., He, B., Shi, X., Wang, L., Liang, J., & Wu, F. (2016). Apigenin and naringenin ameliorate PKCβII-associated endothelial dysfunction via regulating ROS/caspase-3 and NO pathway in endothelial cells exposed to high glucose. *Vascular Pharmacology, 85*, 39-49. doi: 10.1016/ j.vph.2016.07.006Chen, W., Sun, Q., Ju, J., Chen, W., Zhao, X., Zhang, Y., & Yang, Y. (2018). Astragalus polysaccharides inhibit oxidation in high glucose-challenged or SOD2-silenced H9C2 cells. *Diabetes, metabolic syndrome and obesity: targets and therapy, 11*, 673-681. doi: 10.2147/DMSO.S177269
3. Cho, N. H., Shaw, J. E., Karuranga, S., Huang, Y., da Rocha Fernandes, J. D., Ohlrogge, A. W., & Malanda, B. (2018). IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Research and Clinical Practice, 138*, 271-281. doi: 10.1016/j.diabres.2018.02.023
4. Davis, E. F., Lazdam, M., Lewandowski, A. J., Worton, S. A., Kelly, B., Kenworthy, Y., Adwani, S., Wilkinson, A., McCormick, K., Sargent, I., Redman, C., & Leeson, P. (2012). Cardiovascular Risk Factors in Children and Young Adults Born to Preeclamptic Pregnancies: A Systematic Review. *Pediatrics, 129* (6), 1552- 1561. doi: 10.1542/peds.2011-3093

5. Dawish, M. A. A., Robert, A. A., Braham, R., Hayek, A. A., Saeed, A. A., Ahmed, R. A., & Sabaan, F. S. (2016). Diabetes Mellitus in Saudi Arabia: A Review of the Recent Literature. *Current Diabetes Reviews, 12* (4), 359–368. doi: 10.2174/1573399811666150724095130
6. Rajadurai, M., Long, K., Xie, L. M., & Prince, P. (2007). Preventive effect of naringin on cardiac mitochondrial enzymes during isoproterenol-induced myocardial infarction in rats: A transmission electron microscopic study. *Journal of Biochemical and Molecular Toxicology, 21* (6), 354-361. doi: 10.1002/jbt.20203
7. Rampersad, S. N. (2012). Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. *Sensors (Basel, Switzerland), 12* (9), 12347-12360. doi: 10.3390/s120912347
8. Rani, N., Saurabh, B., Bhaskar, K., Jagriti, B., Charu, S., Mohammad Amjad, K., & Dharamvir Singh, A. (2016). Pharmacological Properties and Therapeutic Potential of Naringenin: A Citrus Flavonoid of Pharmaceutical Promise. *Current Pharmaceutical Design, 22* (28), 4341-4359. doi: 10.2174/1381612822666
9. Celiz, G., Daz, M., & Audisio, M. C. (2011). Antibacterial activity of naringin derivatives against pathogenic strains. *Journal of Applied Microbiology, 111* (3), 731-738. doi: 10.1111/j.1365-2672.2011.05070.x
10. Chanet, A., Milenkovic, D., Deval, C., Potier, M., Constans, J., Mazur, A., Bennetau, C., Morand, C., & Bérard, A. M. (2012). Naringin, the major grapefruit flavonoid, specifically affects atherosclerosis development in diet-induced hypercholesterolemia in mice. *The Journal of Nutritional Biochemistry, 23* (5), 469-477. Doi: 10.1016/j.jnutbio.2011.02.001
11. Duncan, J. G. (2011). Mitochondrial dysfunction in diabetic cardiomyopathy. *Biochimica et biophysica acta, 1813* (7), 1351-1359. doi: 10.1016/j.bbamcr.2011.01.014
12. Ekambaram, G., Rajendran, P., Magesh, V., & Sakthisekaran, D. (2008). Naringenin reduces tumor size and weight lost in N-methyl-N′-nitro-N-nitrosoguanidine– induced gastric carcinogenesis in rats. *Nutrition Research, 28* (2), 106-112. doi: 10.1016/j.nutres.2007.12.002
13. Han, X., Yoshizaki, K., Miyazaki, K., Arai, C., Funada, K., Yuta, T., Tian, T., Chiba, Y., Saito, K., Iwamoto, T., Yamada, A., & Fukumoto, S. (2018). The transcription factor NKX2-3 mediates p21 expression and ectodysplasin-A signaling in the enamel knot for cusp formation in tooth development. *The Journal of biological chemistry, 293* (38), 14572-14584. doi: 10.1074/jbc.RA118.00337
14. Hartogh, S. C., Schreurs, C., Monshouwer-Kloots, J. J., Davis, R. P., Elliott, D. A., Mummery, C. L., & Passier, R. (2014). Dual Reporter MESP1mCherry/w- NKX2-5eGFP/w hESCs Enable Studying Early Human Cardiac Differentiation. *STEM CELLS, 33* (1), 56-67. doi: 10.1002/stem.1842
15. Heo, H. J., Kim, M., Lee, J., Choi, S. J., Cho, H., Hong, B., & Shin, D. (2004). Naringenin from *Citrus junos* Has an Inhibitory Effect on Acetylcholinesterase and a Mitigating Effect on Amnesia. *Dementia and Geriatric Cognitive Disorders, 17* (3), 151-157. doi: 10.1159/000076349
16. Galloway, C. A., & Yoon, Y. (2015). Mitochondrial dynamics in diabetic cardiomyopathy. *Antioxidants & redox signaling, 22* (17), 1545-1562. doi: 10.1089/ars.2015.6293
17. Gao, S., Li, P., Yang, H., Fang, S., & Su, W. (2010). Antitussive Effect of Naringin on Experimentally Induced Cough in Guinea Pigs. *Planta Medica, 77* (01), 16-21. doi: 10.1055/s-0030-1250117
18. García-Lafuente, A., Guillamón, E., Villares, A., Rostagno, M. A., & Martínez, J. A. (2009). Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflammation Research, 58* (9), 537-552. doi: 10.1007/s00011-009-0037-3
19. Gay, M. S., Dasgupta, C., Li, Y., Kanna, A., & Zhang, L. (2016). Dexamethasone Induces Cardiomyocyte Terminal Differentiation via Epigenetic Repression of Cyclin D2 Gene. *The Journal of pharmacology and experimental therapeutics, 358* (2), 190-198. doi: 10.1124/jpet.116.234104
20. Goldwasser, J., Cohen, P. Y., Lin, W., Kitsberg, D., Balaguer, P., Polyak, S. J., Chung, R.,

Yarmush, M., & Nahmias, Y. (2011). Naringenin inhibits the assembly and long-term production of infectious hepatitis C virus particles through a PPAR- mediated mechanism. *Journal of hepatology, 55* (5), 963-971. doi: 10.1016/j. jhep.2011.02.011

21. Grosso, C., Valentão, P., Ferreres, F., & Andrade, P. B. (2013). The Use of Flavonoids in Central Nervous System Disorders. *Current Medicinal Chemistry, 20* (37), 4694-4719. doi: 10.2174/09298673113209990155
22. Gu, X., Fang, T., Kang, P., Hu, J., Yu, Y., Li, Z., Cheng, X., & Gao, Q. (2017). Effect of ALDH2 on High Glucose-Induced Cardiac Fibroblast Oxidative Stress, Apoptosis, and Fibrosis. *Oxidative medicine and cellular longevity, 2017*, 1-12. doi: 10.1155/2017/9257967
23. Gul, K., Celebi, A. S., Kacmaz, F., Ozcan, O. C., Ustun, I., Berker, D., Aydin, Y., Delibasi, T., Guler, S., & Barazi, A. O. (2009). Tissue Doppler imaging must be performed to detect early left ventricular dysfunction in patients with type 1
24. diabetes mellitus. *European Journal of Echocardiography, 10* (7), 841-846. doi: 10.1093/ejechocard/jep086
25. Gulsin, G. S., Athithan, L., & McCann, G. P. (2019). Diabetic cardiomyopathy: prevalence, determinants and potential treatments. *Therapeutic advances in endocrinology and metabolism, 10*, 2042018819834869. doi: 10.1177/20420188 19834869
26. Han, X. (2012). Protective effect of naringenin-7-O-glucoside against oxidative stress induced by doxorubicin in H9c2 cardiomyocytes. *BioScience Trends, 6* (1), 19-25. doi: 10.5582/bst.2012.v6.1.19