Original Article

Ameliorative Role of Palmitoleic Acid on Palmitate Induced Lipotoxicity in the Rat Cardiomyocytes

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Received: 28.03.2016 *Accepted: 04.05.2016*

ABSTRACT

Background: Co-supplementation of unsaturated fatty acids (FAs) with saturated FAs may decrease the adverse effects of saturated FA-induced lipotoxicity. The objective of the present study was to evaluate the effect of palmitoleic acid (unsaturated fatty acid) on palmitic acid (saturated fatty acid) induced lipotoxicity criteria in the primary culture of adult rat cardiomyocytes.

Methods: Cells were treated with 0.5 mM palmitic acid, palmitoleic acid, palmitic + palmitoleic acids or remained untreated. The values of cellular triacylglycerol (TAG), diacylglycerol (DAG), DNA fragmentation and cellular viability were evaluated over 24 h, 48 h and 72 h time points.

Results: Co-administration of palmitic and palmitoleic acids increased TAG values over 48 h and 72 h time points compared to the palmitic acid (34.37% and 62.79%, respectively; P <0.001), while decreased DAG values (18.85% and 29.42%, respectively; P <0.01). Moreover, palmitoleic acid decreased DNA fragmentation and increased viability when administrated with palmitic acid (P <0.05).

Conclusion: Palmitoleic acid may be beneficial for diminishing adverse effects of palmitic acid in the rat cardiomyocytes through alterations in the molecule signaling levels.

Keywords: Cardiomyocyte, Lipotoxicity, Palmitic Acid, Palmitoleic Acid, Rat.

IJT 2016 (5): 45-52

INTRODUCTION

The accumulation of extra neutral lipid in the organs such as liver, heart and kidney called lipotoxicity. In the lipotoxic condition, the cell function deteriorates, caused cell death and finally tissue or organ dysfunction [1]. Accordingly, any imbalance between the uptake and utilization of fatty acids is associated with cardiac dysfunction. The extent and duration of metabolic disorders have the detrimental role on the manifestation of lipotoxicity. In the pancrease and heart, where the capacity for regeneration and storage of lipid is low, the extent of organ dysfunction is more obvious. Even little rate of apoptosis in cardiomyocytes leads to organ dysfunction [2].

In this regard, saturated and unsaturated fatty acids (FAs) differ significantly in their contributions to lipotoxicity. Studies on cardiac myocytes [3-5], pancreatic β-cells [6, 7], hepatocytes [8] and breast cancer cell lines [9] showed that lipotoxicity from the accumulation of long chain FAs is specific for saturated FAs. This selectivity has been attributed to the generation of specific proapoptotic lipid species or signaling molecules in response to saturated but not unsaturated FAs. Meanwhile, simultaneous administration of unsaturated FAs with saturated FAs could rescue the saturated FA-induced lipotoxicity. Palmitate, a 16-carbon saturated fatty acid, is one of the most common fatty acids that induce lipotoxicity in various cell types [10]. In this regard, palmitate-induced lipotoxicity has been demonstrated in adult and neonatal rat ventricular myocytes [5, 11-13].

The anticytotoxic effect of palmitoleic acid, a monounsaturated fatty acid with a 16 carbon chain, has been shown in rat hepatoma cells [14]. Besides, palmitoleic acid prevented cell deaths (measured by lactate dehydrogenase release) like oleic acid in neonatal rat cardiomyocytes [5]. To the best of our knowledge the effect of palmitoleic acid on lipotoxicity in the cardiomyocytes has not

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been investigated. Therefore, the purpose of the current study was to evaluate if there is any beneficial effects for palmitoleic acid in the palmitate induced lipotoxicity in the adult rat cardiomyocytes.

MATERIALS AND METHODS

Preparation of Adult Rat Cardiomyocytes

Ventricular heart muscle cells were isolated from 300-350 g male Wistar rats [15] in a slightly modified version. The animal care committee of University of Tehran approved the experimental design and sampling. Briefly, 500 IU heparin sulphate was injected 10 min before anaesthetization and then the rats were anaesthetized with chloroform. The heart with parts of the lung was removed from the thorax after opening it and was soaked into a Petri dish filled with ice-cold physiological NaCl solution. To perfuse, the heart was connected by the aorta to a perfusion system containing carbogenated perfusion buffer for 3-5 min. Then perfusion continued with carbogenated 0.05% trypsin and 0.02 % EDTA in perfusion buffer for 20-25 min. Ventricles were separated from the atria and were cut into small pieces followed by digestion with trypsine-EDTA in perfusion buffer. Cell suspension was filtered through a mesh [size: 200 µm), then centrifuged for 6 min at 25 g. The myocyte-containing pellet was resuspended in perfusion buffer with added 0.2%, 0.4% and 1% $CaCl_2$ (v/v), respectively, and centrifuged again as before mentioned. Finally the pellet was resuspended in BSA gradient (Perfusion buffer, 4% BSA, 1% $CaCl_2$) and centrifuged again at 15 g for 5 min. Ventricular myocytes were seeded into 24-well-plates (about 100,000 cells per well) for 6 h. After that, the wells were washed with PBS and experimental media containing 0.5 mM BSA-conjugated fatty acids was added. Experimental group contain control, palmitic acid, palmitoleic acid and palmitic + palmitoleic acids (0.5 mM of each fatty acid was added simultaneously). Cells were incubated at 37 °C for 72 h. The cell culture medium consisted of medium 199 (Sigma-Aldrich; cat. no. M5017) enriched with 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 100 units/ml penicillin and 100 µg/ml streptomycin.

Lipid Extraction

Lipids were extracted using a modification of the method of Bligh and Dyer [16]. The cardiomyocytes were scraped with 1mL ice-cold methanol, which contained 2% acetic acid. Afterwards, 4 mL of chloroform: methanol (1:2, v/v) was added, and was vortexed vigorously for 15 min, followed by adding 2 mL chloroform and 2 mL of 1 M NaCl solution. The organic lower phase was separated from the upper one by centrifugation and collected by a Pasteur pipette. The lipid extraction was repeated by adding chloroform to the upper layer and centrifugation again. Finally, collected extract was shaken for 30 min and dried under the hood with air and reconstituted in the 0.5 mL chloroform:methanol (2:1). This extract was used to measure triacylglycerol (TAG), diacylglycerol (DAG) and phosphorus.

Determination of Intracellular TAG Concentrations

Intracellular TAG concentrations were measured using a spectrophotometer [17] by slight modification. Briefly, 50 µL of lipid extraction redissolved in 500 µL of choloroform: methanol (2:1). Then 200 µL isopropanol was added to the test tube and the tube was shaken for 1 min. afterwards, 125 µL saponification reagents were added to the test tubes and were incubated at room temperature for 5 min. Each test tube received 250 µl of periodate solution, followed by 250 µl acetylacetone reagent then was incubated at 65 °C for 15 min. After cooling, absorbance of the sample was measured using a spectrophotometer (Shimadzu, Japan) at 410 wave length. Triolein (Sigma-Aldrich; cat. no.T7140) was used as a standard.

Determination of Cellular Diacylglycerol

The organic lower phase derived from lipid extraction was dried, redissolved in 50 µl $CHCl_3$ and spotted on a TLC plate (TLC Silica Gel 60 F254, Merck, Germany) after activation of the plates with methanol. Diacylglycerol was eluted with diethylether/heptane/acetic acid (75:25:1 v:v:v) mixture. The plates were dried and stained with 0.003% Coomassie brilliant blue in 30% CH_3OH and 100 mM NaCl for 30 min. Then, the plates were de-stained for 5 min in dyefree solution and the bands were acquired by Versadoc system. The band densities were calculated using Quantity One software (PowerScan, V.2012). The amount of diacylglycerol was calculated using a standard curve [18].

Determination of Phospholipid (PL)

TAG and DAG values were corrected based on the cellular PL concentration. Lipid

phosphorus accounts for 4% of the total content of phospholipids. Phosphorus levels were measured [19, 20].

Cell Viability Assay

Cell viability was assessed according to Pienata and Lehr [21]. Briefly, in a test tube 1250 µL of the trypan blue solution (0.4%), 750 µL of PBS and 1 µL trypsinized cell suspension were added. The final solution was gently mixed and allowed standing for 3 min at room temperature. Then 10-15 µL of this suspension was loaded onto both chambers of the hemocytometer. More than 200 cells were counted under light microscope at 200x (Zeiss). Viable and nonviable cells were recorded separately and the percentage of cell viability was calculated by the formula: Cell viability (%) = (No. of viable cells (unstained cells)/ Total no. of cells) x 100.

DNA Fragmentation

DNA fragmentation was evaluated with the diphenylamine method [22] but was slightly modified. Cells were scraped and lysed in an appropriate volume of ice-cold lysis buffer (10 mM Tris, 1 mM EDTA (pH 8.0), 0.5% Triton X-100) for 30 min at 4 °C. After lysis, the intact chromatin (pellet with high-molecular-weight DNA) was separated from DNA fragments (supernatant with low-molecular-weight DNA) by centrifugation for 20 min at 27,000 g at 4 °C. Samples were treated with the same volume of trichloracetic acid (TCA) 25% and precipitated for 16 h at 4 °C, and then centrifuged 20 min at 27,000 g at 4 °C and the supernatants were removed. DNA was hydrolyzed by adding the same volume of TCA 5% to each pellet and heating for 15 min at 90 °C. DNA contents were quantitated using the diphenylamine reagent (0.088 M DPA, 98% v/v glacial acetic acid, 1.5% v/v sulfuric acid, 0.5% from 1.6% acetaldehyde solution). Optical density of the two fractions was determined. The percentage of fragmented DNA was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet, considering also the quantity released by cells undergoing apoptosis and lysis during the experiment.

Statistical Analysis

All values are expressed as means ± Standard Error of Mean Statistical analyses for significant differences in DAG, TAG, DNA fragmentation and viability values were assessed using one-way ANOVA procedure (Sigma Stat software 3.1). Alpha was considered at 0.05.

RESULTS

Triacylglycerol Content

Relative TAG values (TAG/PL) were 25.43 ± 0.803, 15.65 ± 0.399, 30.65 ± 1.84 and 24.39 ± 0.76 in control, palmitic, palmitoleic and palmitic + palmitoleic groups, 24 h after incubation, respectively. While palmitic acid made a significant decrease in the cellular TAG (~40%) when compared with control (P<0.001) at different time points, palmitoleic acid restored the values (about 130% and 200% compared to control and palmitic, respectively; P<0.001). However, co-administration of palmitic and palmitoleic acid made a significant decrease in its value in 48 h and 72 h time points (P<0.001), but there were no significant changes in 24 h time point. TAG values were increased in all treated groups at 48 h compared to time 24 h (P<0.001). However, a little decrease was seen in all treated groups at 72 h compared to 48 h, that only was significant in palmitic group (P<0.005). Corrected TAG values were showed in Table 1 and Figure 1.

Table 1. TAG and DAG contents expressed as TAG or DAG / PL in different treatment groups during the culture period.

	TAG/PL			DAG/PL		
	24 h	48 h	72 h	24 h	48 h	72 h
Control	25.46 ± 0.80^{acd}	41.13 ± 1.23^{a}	39.27 ± 0.95^{a}	2.97 ± 0.07^{a}	3.76 ± 0.08^{a}	4.11 ± 0.15^{acd}
Palmitic	15.65 ± 0.39^{b}	32.64 ± 0.80^{b}	23.52 ± 0.71^{b}	5.93 ± 0.19^{b}	6.84 ± 0.15^{b}	7.68 ± 0.52^{b}
Palmitoleic	30.65 ± 1.84^{c}	54.30 ± 0.98^{c}	53.07 ± 0.42^{c}	4.05 ± 0.07^{c}	4.79 ± 0.14^{c}	5.10 ± 0.41^{c}
combination	24.39 ± 0.76^{cd}	43.03 ± 0.73^{ad}	38.29 ± 0.94^{ad}	5.30 ± 0.13^{bd}	5.55 ± 0.07^{cd}	5.42 ± 0.52^{cd}

Data expressed as mean±SE.

Deferent superscript showed significant differences in each column.

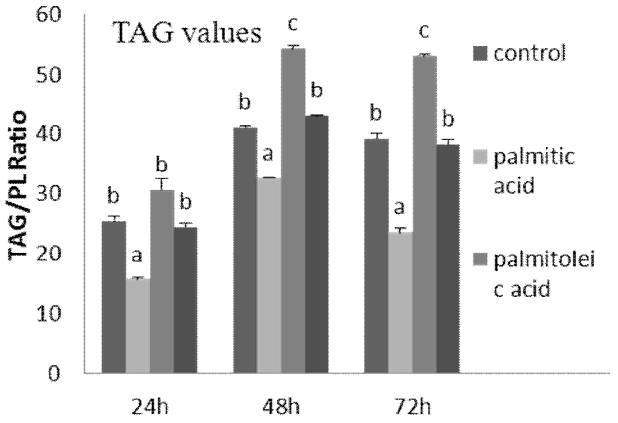

Figure 1. Relative TAG values in different treatment groups and different time points. Between group analyses showed that palmitic acid significantly decreased TAG values compared to control in different time points (P<0.001), while palmitoleic acid increased TAG values significantly compared to palmitic acid and control in the time points (P<0.001). Moreover, co-administration of palmitoleic acid restored the TAG values compared to palmitic acid (P<0.001). Different superscript showed significant differences between groups in a time point.

Diacylglycerol Content

DAG values increased during 24 h to 72 h time points in all treatment groups (P<0.01). Administration of palmitic acid increased about 2 fold in DAG values comparing to the control one (P<0.001), while palmitoleic acid declined the values in comparison with palmitic treated group (P<0.01). However, DAG values were significantly high in all treated groups compared to control in 24 h and 48 h time points (P<0.01). The contents of DAG in control and other treated groups were indicated in Table 1 and Figure 2.

Nuclear DNA Fragmentation

The percentage of DNA fragmentation in palmitic group was 4.11, 3.52 and 1.48 folds more than control, palmitoleic and combination groups, respectively, at 24 h time point. Significant differences were observed between palmitic with other treated groups in all the time points (P<0.01). Palmitic acid enhanced the percent of DNA fragmentation comparing to the control in all the time points (~200%; P<0.01), while administration of palmitoleic to palmitic treated group reduced the fragmentation of DNA compared to palmitic in all the time points (P<0.01). There were no significant differences between palmitoleic and control group at 24 h, 48 h and 72 h time points (P=0.5). The percent of DNA fragmentation in the control and other treated groups were showed in Figure 3.

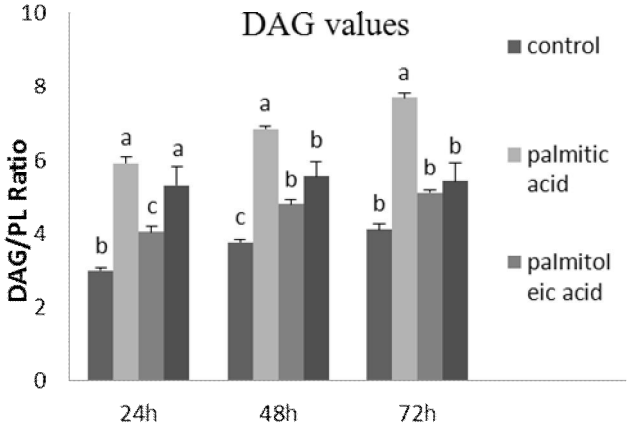

Figure 2. Relative DAG values in different treatment groups and different time points. Between group analyses showed that palmitic acid significantly increased DAG levels compared to the control in the different time points (P<0.01), while palmitoleic acid significantly decreased the DAG values compare to palmitic acid (P<0.01). Simultaneous administration of palmitoleic acid with palmitic acid decreased DAG values significantly at 48h and 72h time points (P<0.01). However, administration of any FFAs increased the DAG level significantly compared to the control at 24h and 48h time points (P<0.02). Different superscript showed significant differences between groups in a time point.

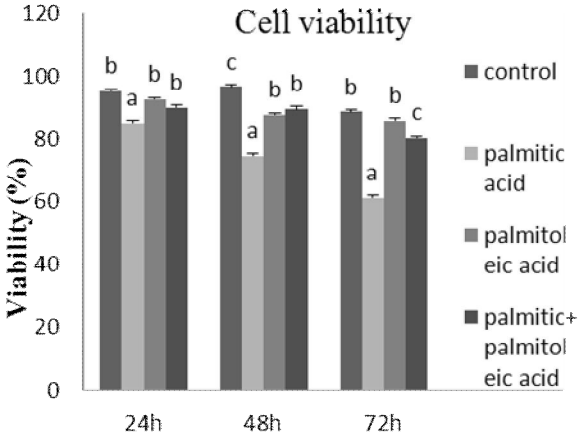

Figure 3. Percent of cell viability in different treatment groups and different time points. Between group analyses showed that palmitic acid significantly increased non-viable cells compared to control in different time points (P<0.001), while palmitoleic acid progressed number of viable cells compared to palmitic acid (P<0.001). Furthermore, co-treatment of palmitoleic acid and palmitic acid increased the percent of viable cells compare to palmitic acid in different time points (P<0.001). Different superscript showed significant differences between groups in a time point.

Cell Viability

Cell viability was significantly lower in palmitic group than other treated groups in all the culture period (P<0.001). Moreover, cell viability did not significantly differ in palmitoleic group (92.71 ± 0.61%) in comparison with control (95.38 ± 0.38%) or combination (90.09 ± 0.81%) group at time 24 h time point. Moreover, the viable cells were significantly higher in palmitoleic + palmitic than palmitic acid in the different time points (P<0.001). The pattern of cell viability changed at 48 h compared to 24 h time point, as all fatty acid treated groups had lower viability than control. Although, differences between control and combination and between palmitoleic and combination were less than 6 percent, this difference was statistically significant (P<0.001) at 72 h time point. The percent of cell viability in control and other treated groups were showed in Figure 4.

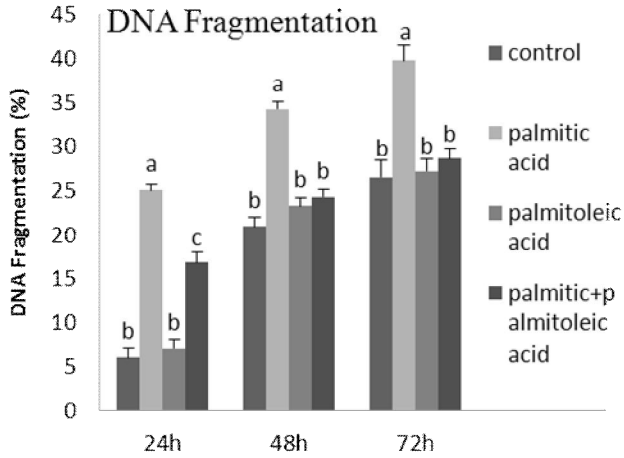

Figure 4. Percent of DNA fragmentation in different treatment groups and different time points. Between group analyses showed that palmitic acid significantly increased the fragmentation of DNA compared to control in different time points (P<0.001), while palmitoleic acid decreased it compared to palmitic acid in different time points (P<0.03). Simultaneous administration of palmitoleic acid and palmitic acid decreased deleterious effect of palmitic acid in different time points (P<0.001). Different superscript showed significant differences between groups in a time point.

DISCUSSION

The co-supplementation with the monounsaturated FA oleate, inhibits the toxicity of the saturated FA palmitate in cardiomyocytes, pancreatic β-cell and CHO cells [5, 13, 23, 24]. In the presence of 0.5 mM oleic acid, the cardiomyocytes remained healthy and accumulated substantial amounts of triacylglycerol [5]. To test that whether palmitoleic acid could increase the formation of TAG stores in cardiomyocytes like oleate [5], when co-treated with palmitate, we measured TAG in the cultured cells. A TAG level in palmitoleate group was near 2 fold of palmitate at 24 h, when palmitoleate co-administreated with palmitate, 60% increase in TAG value was seen in comparison with palmitate at 24 h time point. This pattern was repeated at 48 h and 72 h time points. Our results showed that, palmitoleate promoted TAG formation similar to oleate shown by other studies in different cells [25, 26], especially in the cardiomyocytes [5]. The amount of TAG content decreased at 72 h time point compared to 48 h in all treated groups. Probably, these changes were due to cell death at 72 h time point compared to 48 h and separation of cells from bottom of the wells. Mono Unsaturated Fatty Acid (MUFA) (like oleate or palmitoleate) could promote the detoxification of palmitate by altering its partitioning within the cell. The first was via promotion of fatty acid esterification into TAG such that potentially toxic lipid could safely be stored within lipid droplets. The second was via promotion of its clearance via induction of fatty acid oxidation [27].

Palmitic acid seemed to be incorporated into DAG to a higher extent than oleic acid in myotubes [28], suggesting that various Free Fatty Acids (FFAs) might be utilized differently in the myotubes, which is in line with the study by Montell et al. [25]. Recently, DAG was accumulated in C2C12 myotubes exposed to palmitate and other saturated FFAs but not when exposed to oleic acid [29]. Palmitic acid incorporation into DAG was significantly increased compared with oleic acid incorporation in the myotubes [28]. The extent of saturation of fatty acyl chains in TAG synthesis intermediates, such as phosphatidic acid and DAG, could influence their physicochemical properties, and in this situation could modulate their interactions with specific proteins [30-33]. By increasing the saturated fatty acids in the DAG molecule, the interaction of DAG with protein kinase C would deteriorate. In this situation, instead of lipid crystalline domains, the gel like domains form in

the cell membrane, make this molecule unavailable for the required interactions [31-33]. Moreover, liquid crystalline phase could restore by increasing the unsaturated FAs in DAG, then, making DAG molecules accessible to the interacting proteins [34]. Probably a similar mechanism could satisfactorily explain our results, as well as the majority of previous observations from other laboratories. Besides, there was a difference among unsaturated FAs in DAG to TAG synthesis. The ratio between synthesized triacylglycerol and diacylglycerol was lower in the hepatocytes cultured with Eicosapentaenoic acid (EPA) in the medium compared with Docosahexaenoic acid (DHA) or oleic acid, indicating a decreased conversion of diacylglycerol to triacylglycerol in EPA treated cells [35]. In our experiment, co-supplementation of palmitoleate with palmitate, significantly reduced the concentration of intracellular DAG compared with palmitate alone at 48 h and 72 h time points, showing that palmitoleate could decrease the adverse effect of palmitate and prevented the lipotoxicity by means of increasing DAG. To the best of our knowledge this is the first report of palmitoleate preventive effects on the DAG formation in the rat cardiomyocytes.

A hallmark of apoptosis is the development of numerous cleavages in genomic DNA, specifically-between nucleosomes [36], leading to a typical DNA-fragmentation pattern. In the presence of 0.25 or 0.5 mM palmitate, DNA laddering was observed in the rat cardiomyocytes 16h after culture [5]. The laddering of DNA was prevented by adding of 0.25 mM oleate to 0.25 mM palmitate in their experiment. 0.5 mM palmitate dramatically increased the percentage of Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells in neonatal rat cardiomyocytes, as well [5]. However, in HL-60 cells, there were no significant changes in apoptosis between palmitate (75μM) and oleate (75μM) groups after 48 h [37]. In rat hepatocytes, treatment with 0.25 mM palmitic acid produced a significant increase in cell death [8]. Co-treatment with 0.25 mM alpha linolenic acid reversed the apoptotic effect exerted by palmitic acid [8]. Our results indicated that palmitic acid significantly increased DNA fragmentation like other reports [5, 8], but was not compatible with Declan et al., result (37). However, in the mentioned study the dose of oleic acid probably was low to appear protective effects. Our results revealed that there were no significant differences among control, palmitoleic and combination groups at 48 h and 72 h time points. There were significant increases in the percent of LDH (a cell death marker) released in palmitate and palmitoleate group after 48h compared with the control one [5], opposed to our results. Maybe the neonatal rat cardiomyocytes differed in response to palmitoleate while compared to adult cells. Of course, different results were seen between the adult and neonatal cardiomyocytes in previous studies, for instance, high glucose concentrations prevented apoptosis in the neonatal [38] while did not exert any protective effect in adult rat cardiomyocytes [13]. In our experiment palmitoleic acid significantly declined DNA fragmentation when co-administrated with palmitic acid compared to palmitic alone at 24 h, 48 h and 72 h time points.

In the present experiment, viability of the cardiomyocytes decreased significantly in palmitic group compared to the control and palmitoleic groups. Apoptosis of cardiomyocytes was recently shown to play a crucial role in the development of heart failure [39]. Saturated fatty acid could induce apoptosis in the neonatal rat cardiomyocytes [5, 11, 12]. Cell death was reported after palmitic exposure in different cells in other studies [5, 13, 26, 40]. Oleic acid prevented cell death when simultaneously used with palmitate [5, 26]. The mechanism of palmitate-induced cell death is not completely understood. However, several hypotheses have been advanced to explain palmitate induced cell lipotoxicity. Palmitate is incorporated into de novo synthesis of ceramide [41], a lipid signaling molecule implicated in the induction of apoptosis [42], so that excess palmitate may induce cell death through increased intracellular ceramide concentration [43, 44]. An equally attractive hypothesis, which may especially be relevant to the heart, is that the adverse effect of excess fatty acids is due to metabolic factors; specifically, palmitate may induce cell death by its inability to be completely metabolized, hence, producing partially metabolized fatty acids that are toxic [45].

CONCLUSION

Palmitioleic acid may make an improvement in the rat cardiomyocyte viability and lipotoxicity protection through alteration in the signal transduction pathways.

ACKNOWLEDGEMENT

The authors are grateful to the University of Tehran for providing facilities to conduct and complete this study. The authors declare that there is no conflict of interests.

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