

## Palmitic Acid-Induced Toxicity Reduction Due to Production of Triacylglycerol in Cardiac Muscle Cells by Linoleic Acid

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### ABSTRACT

**BACKGROUND AND OBJECTIVE:** Lipotoxicity-induced cardiovascular diseases are increasing significantly in human populations. The effect of linoleic acid in reducing lipotoxicity of cardiac muscle cells was investigated in this study.

**METHODS:** In this empirical study, ventricular muscle cells from the hearts of five adult rats were cultured in 24-well plates. They were randomly treated in four groups including one control group (0.5 mM bovine serum albumin (BSA)) and three treatment groups treated with palmitic acid (0.5 mM), linoleic acid (0.25 mM) and a combination of linoleic acid and palmitic acid (0.25 mM+0.5 mM). Wells in each row of plates were dedicated to one group. Level of cellular triacylglycerol, cellular diacylglycerol, DNA Fragmentation and survival rate was evaluated 24 and 48 hours after culturing. Each two wells belonging to each group were used to evaluate every factor and all the steps were repeated three times.

**FINDINGS:** Compared with palmitic acid treatment alone, adding linoleic acid to palmitic acid decreased the level of DNA Fragmentation by 4.65% and 6.15% ( $p<0.001$ ) and decreased cellular diacylglycerol content by 0.36% and 4.88% ( $p>0.05$ ). It also increased the level of cellular triacylglycerol by 40% and 44% ( $p<0.03$ ) and increased cellular survival by 6.25% and 10.52% ( $p<0.01$ ), respectively 24 and 48 hours after cultivation.

**CONCLUSION:** Results of the study revealed that linoleic acid reduces the palmitic acid-induced toxicity by producing triacylglycerol.

**KEY WORDS:** *Linoleic Acid, Lipotoxicity, Palmitic Acid, Rat Cardiac Muscle, Triacylglycerol, Diacylglycerol.*

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## Introduction

Cardiac muscle cells have limited capacity to synthesize fatty acids, so they have to receive fatty acids from blood circulation (1). However, the imbalance between the capacity to receive these fatty acids and the ability to use them leads to accumulation of lipids (2-4). Studies have shown that increase in the level of free fatty acids decreases the oxidation of fatty acid and increases the synthesis of toxic lipids such as diacylglycerol in cardiac muscle tissue (5). Increase in the level of free fatty acids and accumulation of lipids is called lipotoxicity. Lipotoxicity in myocytes can cause cellular disorders, incomplete expansion and contraction or both, redirection of key signals and cellular death (6).

There are different opinions about the effect of fatty acids on occurrence and prevention of lipotoxicity. Presence of one or more double bond in the composition of unsaturated fatty acids can show various degrees of resistance against lipotoxicity (7,8). Increase in the level of saturated fatty acids increases the production of free radicals, weakens antioxidant defense system and eventually induces apoptosis (9,10). Fatty acids-induced apoptosis and lipotoxicity in endothelial cells (11), skeletal cells (12) and HIT-T15 cells (13) were studied. While unsaturated fatty acids had antagonizing effects on saturated fatty acids-induced lipotoxicity (14,15), palmitic acid and stearic acid caused apoptosis and cellular death in neural cells, but oleic acid and arachidonic acid did not have such effects on neural cells (16). In the study of Cheon et al., palmitic acid increased cellular death in a dose-dependent manner. However, arachidonic acid controlled the palmitic acid-induced lipotoxicity with a density of 62  $\mu$ M (17).

In addition, protective effects of unsaturated fatty acids have been found in cardiac muscle cells and hepatocytes (18, 19). It seems that accumulation of palmitic acid alone or other intermediate signals such as diacylglycerol, which are the result of such metabolites, can be responsible for inducing apoptosis in cardiac muscle cells (20-22). So far, no comprehensive study has been conducted on the effect of linoleic fatty acid (n-6) on cardiac muscle cells against palmitic acid. Therefore, studying the effect of these fatty acids on indicators of lipotoxicity in cardiac muscle cells can be a huge step forward for discovering the effect of this fatty acid on the aforementioned cardiac muscle cells and possibly the effect of this fatty acid in decreasing the rate of

lipotoxicity-induced heart diseases. Hence, the effect of linoleic acid in decreasing lipotoxicity in cardiac muscle cells was investigated in this study.

## Methods

**Isolating and culturing adult rat ventricular cardiac muscle cells:** In this empirical study, adult rat cardiac muscle cells were isolated and cultured using Piper and Volz method (23). Cells isolated from the heart of each rat were counted and transferred to each well of the 24-well plates along with M-199 Medium supplemented with taurine, creatine, carnitine and 4% FCS (Fetal Calf Serum) in desired numbers (about 100000 cells). In other words, cardiac muscle cells of each rat were transferred to a 24-well plate (cells were distributed equally in each well) and incubated for 5 hours. Cells in each well were then washed in a washing buffer containing calcium and magnesium and wells were divided randomly in four groups including one control group (0.5 mM bovine serum albumin (BSA)) and three treatment groups treated with palmitic acid (0.5 mM), linoleic acid (0.25 mM) and combination of linoleic acid and palmitic acid (0.25 mM+0.5 mM). Wells in each row of plates were dedicated to one group. Each two wells of each group were used to evaluate each variable and all the steps were repeated three times.

Five rats were used in this study totally. It is worth noting that all of the fatty acids were used with bovine serum albumin conjugates. Cells were cultured in incubator (containing 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C) for 48 hours and sampling was done 24 and 48 hours after culturing to evaluate the following variables (level of cellular triacylglycerol, cellular diacylglycerol, DNA Fragmentation and survival rate).

**Cellular lipid extraction:** Cellular lipid was extracted according to the Bligh and Dyer proposed method of extraction (24). Cells in each well were scratched in the presence of methanol and acetic acid. They were then combined with methanol/chloroform and after adding chloroform, 2 mL of sodium chloride solution was added and they were centrifuged in the end. The lower phase was collected. Then the contents of pipes were aerated and 0.5 mL of methanol/chloroform was added to each one.

**Measuring cellular triacylglycerol:** Cellular triacylglycerol was measured according to the proposed method of Neri and Frings (25). Based on this method, light absorption of samples at 410 nm was

read using isopropanol, soap solution, peridot solution and acetyl acetone reagent.

**Measuring cellular diacylglycerol:** Cellular diacylglycerol was measured according to the proposed method of Baldanzi (26). Silica gel plates (Thin layer chromatography Silicagel 60 F254, Merck) were activated with methanol. After staining, plate was put in the tank which contained solution and was dyed in the end. Stains in each group were compared with standard diacylglycerol stains using PowerScan 2012 Software.

**Measuring total cellular protein:** Cellular protein was measured according to the proposed method of Bradford (27). In this method, level of cellular protein with a wavelength set at 595 nm was read using Bradford reagent and standards obtained from bovine serum albumin (BSA).

**Evaluating cellular DNA fragmentation:** Cellular DNA fragmentation was evaluated according to the proposed method of Sandau [28]. Cells in each well were transferred to separate microtubes. Each well was washed with 500  $\mu$ L of lysis buffer and was added to the cells in microtube and was kept at 4°C. Supernatant fluid (s) was separated from pellet (p) and was poured into another microtube and trichloroacetic acid was added to both of them and they were finally stored at 4°C. In order to hydrolyze DNA, 5% trichloroacetic acid was added to the pellet of s and the pellet of p and they were put in bain-marie at 90°C for 15 minutes. Finally, diphenylamine (DPA) reagent was added to them and 12-24 hours later, OD of s and p with a wavelength set at 600 nm was read.

$$\text{DNA fragmentation \%} = [\text{OD s} / (\text{OD p} + \text{OD s})] \times 100$$

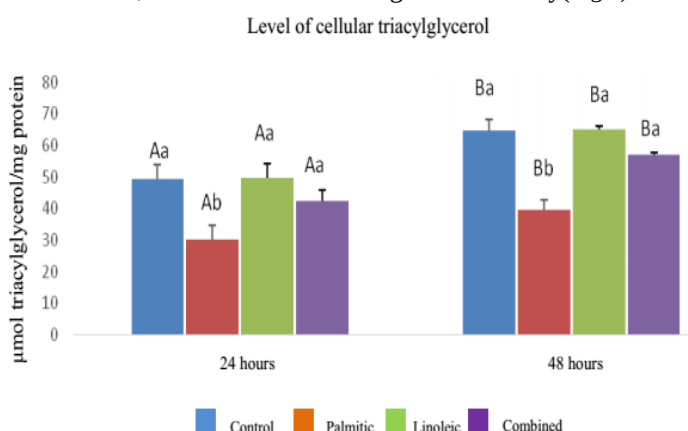
**Evaluating cellular survival:** Cellular survival was evaluated according to the proposed method of Pienata and Lehr [29]. Available data was analyzed with SigmaStat Software (version 3.5), statistical test of ANOVA between multiple groups and statistical test of T-Test in one group after 24 and 48 hours ( $p < 0.05$ ).

## Results

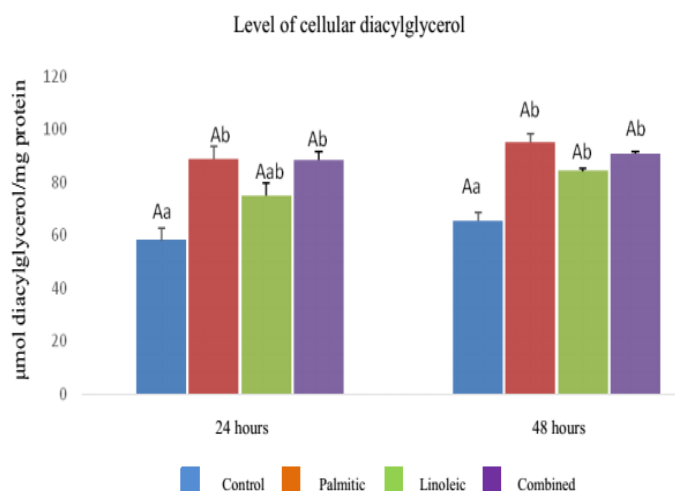
**Cellular triacylglycerol:** After 24 and 48 hours, cellular triacylglycerol in palmitic acid group was less than other groups ( $p < 0.004$ ). Moreover, after adding linoleic acid to palmitic acid in combined group, level of triacylglycerol showed a meaningful increase compared to the palmitic acid group ( $p < 0.02$ ), but it did not show a meaningful difference with the level of

triacylglycerol in linoleic acid group and control group ( $p > 0.05$ , Fig 1). After 48 hours, level of triacylglycerol in control group, palmitic acid group, linoleic acid group and combined group was 64.68, 39.48, 65.04 and 56.97  $\mu$ mol/mg protein, respectively, which showed a meaningful increase (49.30, 30, 49.55 and 42.40  $\mu$ mol/mg protein) compared to the same groups after 24 hours ( $p < 0.03$ , Fig 1).

**Cellular diacylglycerol:** Level of cellular diacylglycerol based on  $\mu$ mol/mg protein was lowest in control group (58.09  $\mu$ mol/mg protein) and highest in palmitic acid group (88.63  $\mu$ mol/mg protein) after 24 hours. Moreover, level of cellular diacylglycerol in linoleic acid group (74.88  $\mu$ mol/mg protein) was higher than control group and lower than palmitic acid group (88.63  $\mu$ mol/mg protein) and combined group (88.31  $\mu$ mol/mg protein) but there was no meaningful difference with other groups ( $p > 0.05$ ). Level of cellular diacylglycerol in combined group was somewhere between the level of cellular diacylglycerol in palmitic acid group and linoleic acid group, but no meaningful difference was witnessed. After 48 hours, only the level of cellular diacylglycerol in control group (65.39  $\mu$ mol/mg protein) revealed a meaningful difference with other groups. At this moment, level of cellular diacylglycerol in palmitic acid group (95.21  $\mu$ mol/mg protein) was higher than combined group (90.78  $\mu$ mol/mg protein) and linoleic acid group (84.16  $\mu$ mol/mg protein), but no meaningful difference was witnessed (Fig 2). Level of cellular diacylglycerol in control group, palmitic acid group, linoleic acid group and combined group was higher after 48 hours compared to the level of cellular diacylglycerol after 24 hours, which was not meaningful statistically (Fig 2).



**Figure 1. Level of cellular triacylglycerol in primary culture of adult rat cardiac muscle cells in control group, palmitic acid group, linoleic acid group and combined group after 24 and 48 hours**



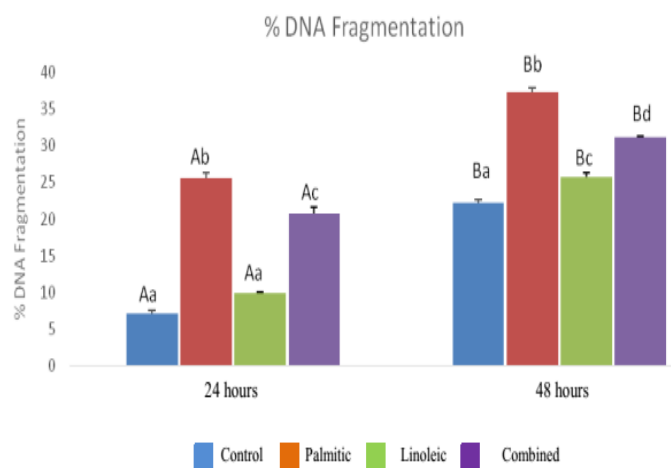
**Figure 2. Level of cellular diacylglycerol in primary culture of adult rat cardiac muscle cells in control group, palmitic acid group, linoleic acid group and combined group after 24 and 48 hours.**

**DNA fragmentation:** After 24 and 48 hours, palmitic acid increased the level of DNA fragmentation compared to other groups ( $p < 0.001$ ); while adding linoleic acid to palmitic acid (combined group) decreased the level of DNA fragmentation by 5 and 6 percent after 24 and 48 hours, respectively. After 24 hours, percentage of DNA fragmentation in control group and linoleic acid group was 7.9 and 9.95, respectively, which revealed no meaningful difference statistically; but after 48 hours, this percentage was 22.25 and 25.75, respectively, which revealed a meaningful difference statistically ( $p < 0.001$ , Fig 3). Percentage of DNA fragmentation in control group, palmitic acid group, linoleic acid group and combined group increased meaningfully after 48 hours compared to percentage of DNA fragmentation after 24 hours ( $p < 0.001$ , Fig 3).

**Cellular survival:** After 48 hours, percentage of cellular survival in palmitic acid group and linoleic acid group (treatment groups) was 71.25 and 83.15, respectively, revealing a meaningful decline compared to the percentage of cellular survival after 24 hours (79.25 and 88.15, respectively) ( $p = 0.003$ ); but this decline was not meaningful in control group and combined group (Fig 4).

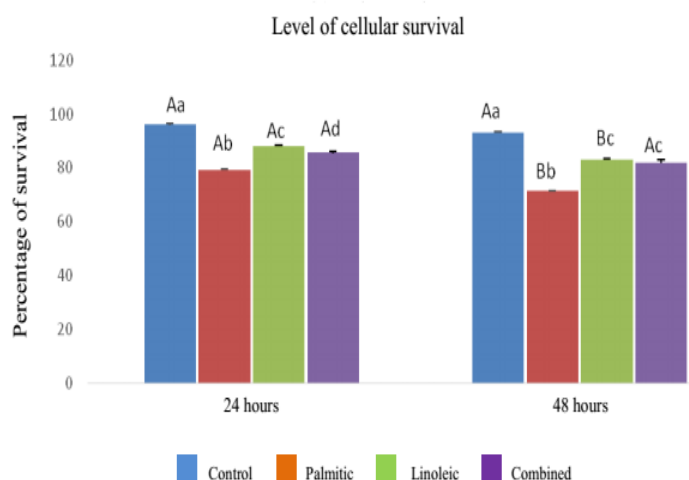
Percentage of cellular survival in palmitic acid group after 24 and 48 hours was lower than other groups ( $p < 0.01$ ). At the aforementioned moments, percentage of living cells in combined group was 85.5 and 81.77, respectively, which was higher than palmitic acid group ( $p < 0.01$ ); also, this percentage was higher in control group compared to linoleic acid group ( $p < 0.001$ ). Percentage of cellular survival in

combined group after 24 and 48 hours was lower than linoleic acid group, but this difference was only meaningful after 24 hours ( $p < 0.02$ , Fig 4).



**Figure 3. Percentage of DNA fragmentation in primary culture of adult rat cardiac muscle cells in control group, palmitic acid group, linoleic acid group and combined group after 24 and 48 hours.**

a, b and c show the statistical differences between groups (control or treatment) at a specific time (after 24 hours and 48 hours), while A and B show statistical differences in each group (control or treatment) after 24 hours compared to 48 hours and similar letters indicate lack of a meaningful difference.



**Figure 4. Percentage of cellular survival in primary culture of adult rat cardiac muscle cells in control group, palmitic acid group, linoleic acid group and combined group after 24 and 48 hours.**

a, b and c show the statistical differences between groups (control or treatment) at a specific time (after 24 hours and 48 hours), while A and B show statistical differences in each group (control or treatment) after 24 hours compared to 48 hours and similar letters indicate lack of a meaningful difference.

**Total protein:** Level of protein in adult rat cardiac muscle cells in control group, palmitic acid group, linoleic acid group and combined group after 48 hours of incubation is  $2.27 \pm 0.27$ ,  $2.72 \pm 0.06$ ,  $2.07 \pm 0.19$  and  $2.37 \pm 0.05$  ug/mL, respectively.

## Discussion

In the present study, co-administration of palmitic acid and linoleic acid in cardiac muscle cells increased the level of triacylglycerol meaningfully compared to palmitic acid group after 24 and 48 hours. The protective effect of unsaturated fatty acids in preventing lipotoxicity of palmitic acid is due to conversion of palmitate to triacylglycerol (30). Similar studies have shown that unsaturated fatty acids create lipid droplets in the cytosol of C2C12 cells, while saturated fatty acids alone cannot create lipid droplets (17). The biological effect of triacylglycerol accumulation on lipotoxicity of palmitic acid is controversial (31). Triacylglycerol accumulation in pancreatic beta cells and impaired insulin secretion are closely related (31). On the other hand, it has been reported that fatty acids, which have a high capacity to synthesize triacylglycerol, participate in prevention of palmitic acid-induced apoptosis by decomposing palmitic acid into triacylglycerol stores (32). Cnop et al. have shown that there is an inverse relationship between percentage of dead pancreatic beta cells and content of their cellular triacylglycerol (33).

Also, it has been reported that unsaturated fatty acids protect palmitic acid-induced apoptosis in nonfat cells by involving palmitic acid in the process of triacylglycerol production. This accumulation is in fact a primary cellular defense against lipotoxicity (22). Furthermore, a mechanism that inhibits undesirable effects of saturated fatty acid by unsaturated fatty acid is a decline in palmitate accumulation in the form of diacylglycerol (DAG) (34). In the present study, co-administration of palmitic acid and linoleic acid in cardiac muscle cells led to a decline in the level of diacylglycerol compared to palmitic acid group after 24 and 48 hours, though it was not statistically meaningful. Division and fragmentation of DNA is one of the signs of apoptosis (35). Division of DNA

eventually creates the pattern of DNA fragmentation. Unsaturated fatty acids have antagonizing effects on fatty acid-induced lipotoxicity (36). Treating human beta cells with unsaturated fatty acid prevented saturated fatty acid-induced apoptosis completely (36). De Vries et al. have reported that treating rat ventricular cardiac muscle cells with palmitate for 16 hours induced meaningful DNA fragmentation compared to control group (19).

Although no meaningful difference was witnessed in the level of apoptosis in HD-60 cell line between palmitate group and oleate group after 48 hours (37), treatment with palmitic acid in rat hepatocytes led to a meaningful increase in cell death (38). Adding linoleic acid to palmitic acid prevented palmitate-induced apoptosis (38). Moreover, palmitate increased DNA fragmentation in C2C1 cells at 0.5 mM density (17). The present study revealed that adding linoleic acid to palmitic acid led to a meaningful decline in DNA fragmentation compared to palmitic acid group. Cellular survival after 24 and 48 hours in palmitic acid group was less than the other three groups. However, after adding linoleic acid to palmitic acid, percentage of live cells was more than palmitic acid group after 24 and 48 hours. Palmitic acid induces cell death and apoptosis (15, 11). Apoptosis due to treatment with saturated fatty acids was shown in different cells (18, 19). The mechanism that plays a key role in creation of palmitate-induced apoptosis in cardiac muscle cells is the induction of synthesis of ceramides (39). A study has shown that oleic acid plays a protective role against inflammation and cell death compared to saturated fatty acids (40).

Results of the present study showed that co-administration of palmitic acid and linoleic acid prevents the toxic effects of palmitic acid on cardiac muscle cells. This is the first report on positive effects of linoleic acid on adult rat ventricular cardiac muscle cells in preventing lipotoxicity.

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