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Toxicity of fatty acids on ECV-304 endothelial cells

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ABSTRACT

The effects of stearic (saturated) or oleic (monounsaturated) acids and their combination with ω-3 and ω-6 polyunsaturated fatty acids (PUFA) on death of endothelial cells (ECV-304 cell line) were investigated. We examined: loss of plasma membrane integrity, DNA fragmentation, accumulation of neutral lipids (NL) and release of reactive oxygen species (ROS). The fatty acids studied were: stearic (SA), oleic (OA), docosahexaenoic (DHA), eicosapentaenoic (EPA), linoleic (LA) and gamma-linolenic (γA) acids. SA at 150 μM induced cell death, did not lead to accumulation of NL and raised the release of ROS. ω-3 PUFA decreased ROS production, increased NL content but did not protect against ECV-304 cell death induced by SA. ω-6 PUFA inhibited SA-induced cell death, increased NL content and decreased ROS production. OA caused cell death but did not increase NL content and ROS production even at 300 μM. ω-3 and ω-6 FA associated with OA further increased cell death with no change in ROS production and NL content. Concluding, ω-6 PUFA had a greater protective effect than ω-3 PUFA on the deleterious effects caused by SA whereas OA had low cytotoxicity but, when associated with PUFA, presented marked toxic effects on ECV-304 endothelial cells.

1. Introduction

Obesity and high fat diets promote increased plasma concentrations of free fatty acids (FFA) leading to endothelial dysfunction (Mattern and Hardin, 2007). The most abundant FFA in plasma are stearic (SA), palmitic (PA) (saturated) and oleic (OA) (monounsaturated) acids (Hagenfeldt et al., 1972). Saturated FA have pro-inflammatory actions (Basu et al., 2006) and increase the risk of cardiovascular diseases (CVD) (Oh et al., 2005; Singh et al., 2002), whereas monounsaturated FA have been associated with a reduced risk of cardiovascular diseases (West and York, 1998). ω-3 Polyunsaturated FA (PUFA; EPA and DHA) present anti-inflammatory effects and decrease the release of pro-atherosclerotic factors (He et al., 2009), whereas the effects of ω-6 PUFA (e.g. linoleic and γ-linolenic acid) in the prevention of CVD still remain controversial (Harris, 2008; Lecerf, 2009). High concentrations of FFA cause apoptosis and necrosis in lymphocytes (Gorjão et al., 2007), macrophages (Cury-Boaventura et al., 2006a) and neutrophils (Cury-Boaventura et al., 2006b; Hatanaka et al., 2006). In spite of this information, the effect of FA on endothelial cell (EC) death was poorly investigated. The sites where plaques develop are associated with increased EC turnover rate due to the occurrence of cell death (Xu, 2009). Endothelial microparticles are increased in patients with unstable coronary disease, and account for procoagulant activity of the plaque (Tan et al., 2005). This information led us to investigate the effect of FA on EC death. We studied the effects of the most abundant fatty acids in the diet (stearic, oleic, linoleic and γ-linolenic acids) and ω-3 PUFA (EPA and DHA) that has being used as therapeutic agents in several pathological conditions (e.g. atherosclerosis and autoimmune diseases). We examined if ω-3 and ω-6 PUFA can protect EC from death induced by SA that is highly cytotoxic for several cell types (Harvey et al., 2010; De Lima-Salgado et al., 2011). ω-3 and ω-6 PUFA was also tested in combination with OA that presents low cytotoxicity (de Lima et al., 2006; Levada-Pires et al., 2010). Neutral lipids (NL) and ROS contents were also determined.

2. Materials and methods

2.1. Culture conditions

ECV-304 is a unique spontaneously transformed human umbilical vein endothelial cell and has several practical advantages over others endothelial cell lines such as an enhanced and highly reproducible capacity for in vitro angiogenesis (Mutin et al., 1997). Besides that, human EC line ECV-304 was characterized and compared with human umbilical vein EC endothelial cell markers (Hughes, 1996; Mutin et al., 1997; Wang et al., 2011). ECV-304 cells were maintained in RPMI-1640 culture medium containing

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10% fetal bovine serum (FBS) supplemented with glutamine (2 mM), HEPES (20 mM), streptomycin (10,000 g/mL) and sodium bicarbonate (24 mM). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were treated with SA or OA combined with LA, γA, EPA or DHA dissolved in ethanol. The concentrations used were based on preliminary studies. We used toxic concentrations of SA (150 μM) and OA (300 μM) acids. PUFA (ω-3 and ω-6) were used at 50 and 100 μM.

2.2. Cytotoxicity determination

Signs of cell death (cell viability and DNA fragmentation) were evaluated in a time course study carried out for 2, 6 or 24 h after FA treatment. The proportion of cells with loss of membrane integrity and fragmented DNA was determined by flow cytometry using a FACSCalibur equipment (Becton and Dickinson System, San Juan, California, USA), as previously described (Jaroszeski and Radcliff, 1999; de Lima et al., 2007).

2.3. Oil red O staining

ECV-304 cells were treated with FA for 24 h, than the slides were washed, fixed and stained with oil red O as previously described (Pearse, 1960). The slides were examined by light microscopy at 510 nm (Carl Zeiss Vision, Munchen-Hallbergmoos, Germany). Images were taken at 20× magnification and a representative image is shown (Figs. 2C and 4C).

2.4. Measurement of ROS

Cells were treated with the FA for 30 min. After treatment, the cells were incubated with hydroethydine (1 μM) for 30 min at room temperature in the dark. Cells were visualized in a fluorescence microscope (Carl Zeiss Vision, Munchen-Hallbergmoos, Germany), using the 590/46 nm filter and analyzed by fluorescence intensity using the KS 300 software. For quantification of ROS production images were taken at 20× magnification from 10 random fields of view for each well and were analyzed by fluorescence intensity using the KS 300 software. Values of the areas were averaged to obtain the mean values. A representative image is shown (Figs. 2D and 4D).

2.5. Statistical analysis

Results are presented as means ± SEM of 6–9 determinations from 2 to 3 experiments. Statistical analysis was performed by using one-way ANOVA and Tukey's test (Graph Pad Prism 5; Graph Pad software) as indicated. The level of significance was set at $p < 0.05$.

3. Results

3.1. Combined effects of SA with ω-3 and ω-6 PUFA

Treatment with SA for 24 h decreased the proportion of viable cells by 18% at 150 μM, 9% at 200 μM and 11% at 250 μM, as compared to vehicle (Fig. 1A). The proportion of cells with DNA fragmentation was increased by 3-fold due to treatment with SA at 150 μM, by 3.5-fold at 200 μM and 4-fold at 250 μM for 24 h, as compared to vehicle (Fig. 1B). The treatment with SA at 150 and 200 μM for 24 h did not change the content of lipids but at 250 μM decreased it by 25% compared to vehicle (Fig. 1C). ROS Production was increased by approximately 2-fold due to SA

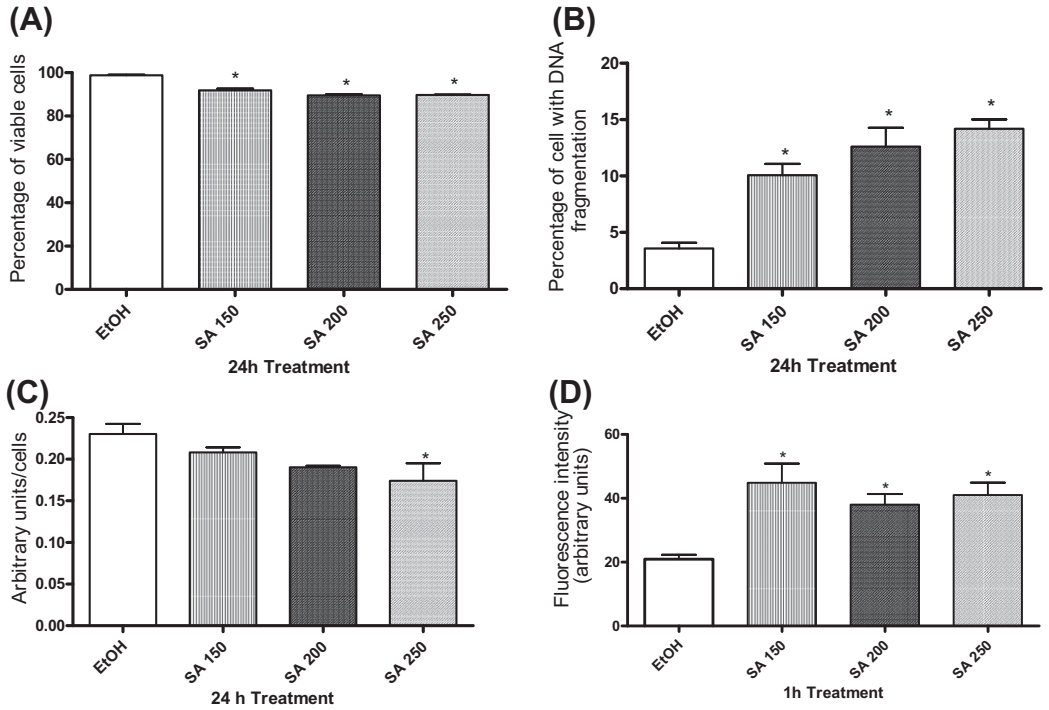

Fig. 1. ECV-304 endothelial cells incubated with stearic acid for 24 h. (A) Cell viability; (B) DNA fragmentation: ECV-304 endothelial cells were incubated with fatty acids for 24 h and analyzed by flow cytometry. The fluorescence was measured in FL2 channel (585/42 nm). (C) Oil red O staining: ECV-304 endothelial cells were incubated with fatty acids for 24 h and then were washed, fixed and stained with oil red O. The cells were examined by light microscopy and by absorbance assay at 510 nm. (D) Fluorescence intensity relative to the content of reactive oxygen species: ECV-304 endothelial cells were incubated with fatty acids for 30 min and then with hydroethydine for 30 min. Subsequently, the cells were analyzed to determine the production of superoxide anion in a fluorescence microscope using 590/46 nm filter. (D) The graph shows the values of production of ROS induced by fatty acid treatment subtracted of the values obtained with ethanol. Values are presented as mean ± SEM ($n = 9$). $*$ $p < 0.05$ compared to ethanol as indicated by ANOVA, $^{\#}p < 0.05$ compared to stearic acid as indicated by ANOVA. EtOH = ethanol; SA 150 = stearic acid at 150 μM; SA 200 = stearic acid at 200 μM; SA 250 = stearic acid at 250 μM.

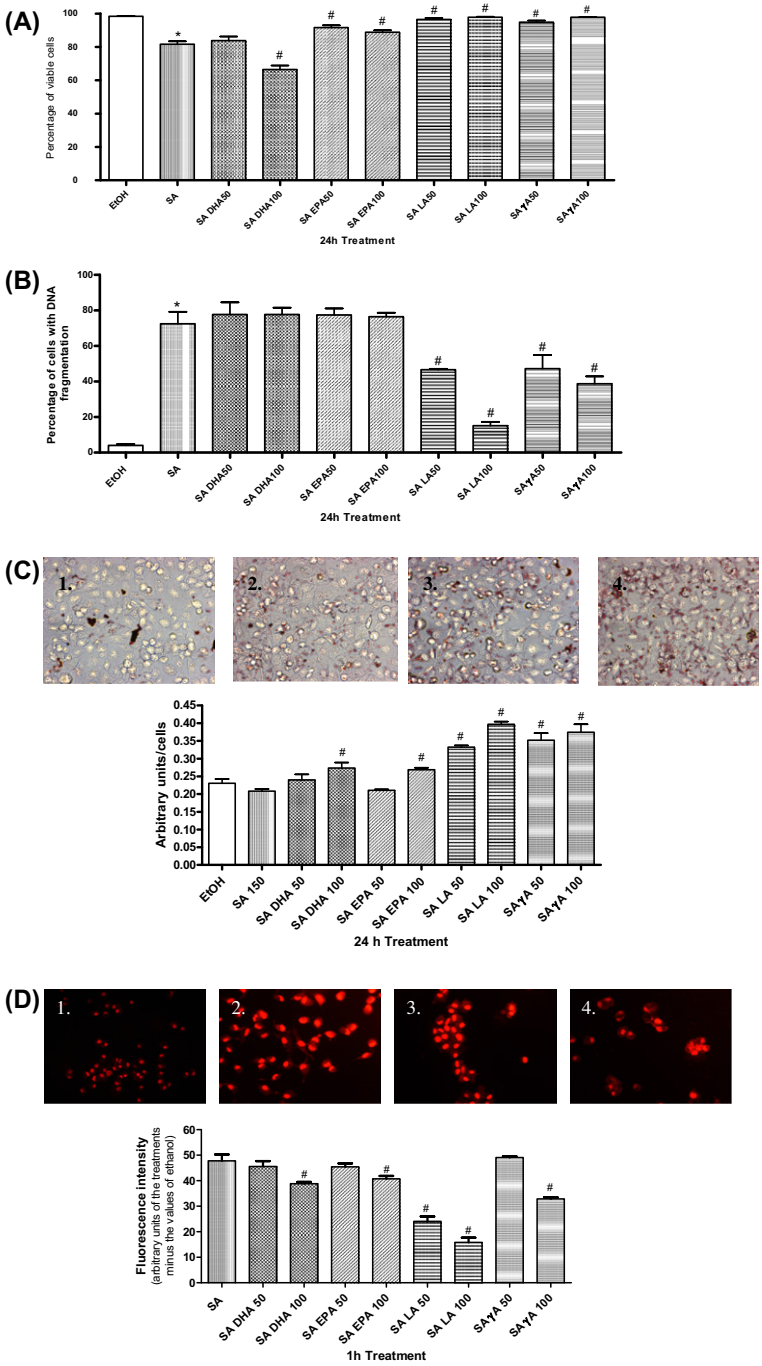

Fig. 2. ECV-304 endothelial cells incubated with stearic acid combined with ω-6 or ω-3 fatty acids for 24 h. (A) Cell viability; (B) DNA fragmentation: ECV-304 endothelial cells were incubated with fatty acids for 24 h and analyzed by flow cytometry. The fluorescence was measured in FL2 channel (585/42 nm). (C) Oil red O staining: ECV-304 endothelial cells were incubated with fatty acids for 24 h and then were washed, fixed and stained with oil red O. The cells were examined by light microscopy and by absorbance assay at 510 nm. (D) Fluorescence intensity relative to the content of reactive oxygen species: ECV-304 endothelial cells were incubated with fatty acids for 30 min and then with hydroethydine for 30 min. Subsequently, the cells were analyzed to determine the production of superoxide anion in a fluorescence microscope using 590/46 nm filter. (C, D) The photos illustrate the most significant results; 1. ethanol; 2. stearic acid 150 μM; 3. stearic acid 150 μM plus eicosapentaenoic acid 100 μM; 4. stearic acid 150 μM plus linoleic acid 100 μM; (D) The graphs show the results of fluorescence intensity of the treatments minus the values obtained with ethanol, expressed as mean ± SEM of three experiments performed in duplicate. $^{*}p < 0.05$ compared to ethanol as indicated by ANOVA, $^{\#}p < 0.05$ compared to stearic acid as indicated by ANOVA. EtOH = ethanol; SA = stearic acid always at 150 μM; DHA = docosaexaenoic acid; EPA = eicosapentaenoic acid; LA = linoleic acid; γA = γ-linolenic acid; all at 50 or 100 μM as indicated.

treatment either at 150, 200 and 250 μM, as compared to vehicle (Fig. 1D).

Treatment with SA and the association with PUFA (ω-3 and ω-6) for 2 and 6 h did not alter the viability and the percentage of cells with DNA fragmentation compared to vehicle (data not shown). Treatment with SA for 24 h decreased the proportion of viable cells by 18% at 150 μM as compared to vehicle (Fig. 2A). The combination of SA with DHA at 100 μM decreased still further the proportion of viable cells by 19% as compared to SA. On the other hand, the association of SA with EPA at 50 and 100 μM increased the proportion of viable cells by 12% and 9%, respectively, compared to SA. ω-6 FA (LA and γA, at 50 and

100 μM) increased the proportion of viable cells in the presence of SA by 20% as compared to SA (Fig. 2A).

The proportion of cells with DNA fragmentation was increased by 18-fold due to treatment with SA at 150 μM for 24 h as compared to vehicle (Fig. 2B). ω-3 PUFA did not alter the SA effect. However, ω-6 PUFA markedly reduced the proportion of cells with DNA fragmentation caused by the SA. The reduction in the proportion of cells with DNA fragmentation induced by ω-6 PUFA was as follows: by 36% and 79% for LA at 50 and 100 μM, respectively, and by 35% and 47% for γA at 50 and 100 μM, respectively, all compared to SA (Fig. 2B).

Treatment with ω-3 PUFA (DHA and EPA, both at 100 μM) associated with SA at 150 μM for 24 h increased NL content by 31% and 29%, respectively, both compared to SA. The increased NL content induced by ω-6 PUFA was as follows: by 60% and 91% for LA at 50 and 100 μM, respectively, and by 69% and 80% for γA at 50 and 100 μM, respectively, all compared to SA (Fig. 2C).

The content of ROS in FA treatments (Fig. 2D) were subtracted of the values obtained with the vehicle. ROS Production was increased by approximately 2-fold due to SA treatment at 150 μM (Fig. 2D). SA associated with DHA, EPA and γA at 50 μM did not alter the ROS production compared to SA. However, combinations of SA with DHA, EPA and γA at 100 μM decreased by approximately 20% the ROS production compared to SA. SA plus LA at 50 and 100 μM decreased by 50% and 67%, respectively, the ROS content compared to SA (Fig. 2D).

3.2. Combined effects of OA with ω-3 and ω-6 PUFA

OA at 300, 350 and 400 μM for 24 h did not alter the integrity of plasma membrane compared to vehicle (Fig. 3A). The treatment with OA for 24 h increased the proportion of cells with DNA fragmentation by 5-fold at 300 μM, by 8-fold at 350 μM and by 10-fold at 400 μM, compared to vehicle (Fig. 3B). The NL content was decreased by 68% with OA at 300, 350 and 400 μM (Fig. 3C). OA at 300 and 350 μM did not alter ROS production but at 400 μM increased by 50% as compared to vehicle (Fig. 3D).

Treatment with OA at 300 μM only or associated with ω-3 FA for 2 and 6 h did not alter the cell viability and fragmentation of DNA as compared to vehicle. However, OA associated with ω-6 FA for 6 h reduced the proportion of viable cells by 49% and 57% for LA at 50 and 100 μM, respectively, and by 52% for γA at 100 μM, as compared to OA (data not shown). The fragmentation of DNA was increased by the association of OA with ω-6 FA for 6 h by 8- and 16-fold for LA at 50 and 100 μM, respectively; and by 5- and 16-fold for γA at 50 and 100 μM, respectively (data not shown).

OA at 300 μM for 24 h did not alter the integrity of plasma membrane compared to vehicle (Fig. 4A). On the other hand, OA associated with ω-3 and ω-6 PUFA for 24 h reduced cell viability by: 87% and 91% for DHA; 81% and 87% for EPA; 76 and 77% for LA; 75 and 83% by γA, all at 50 and 100 μM, respectively (Fig. 4A).

The treatment with OA at 300 μM for 24 h increased the proportion of cells with DNA fragmentation by 5-fold (Fig. 4B). The combination of OA with ω-3 and ω-6 PUFA increased this parameter still

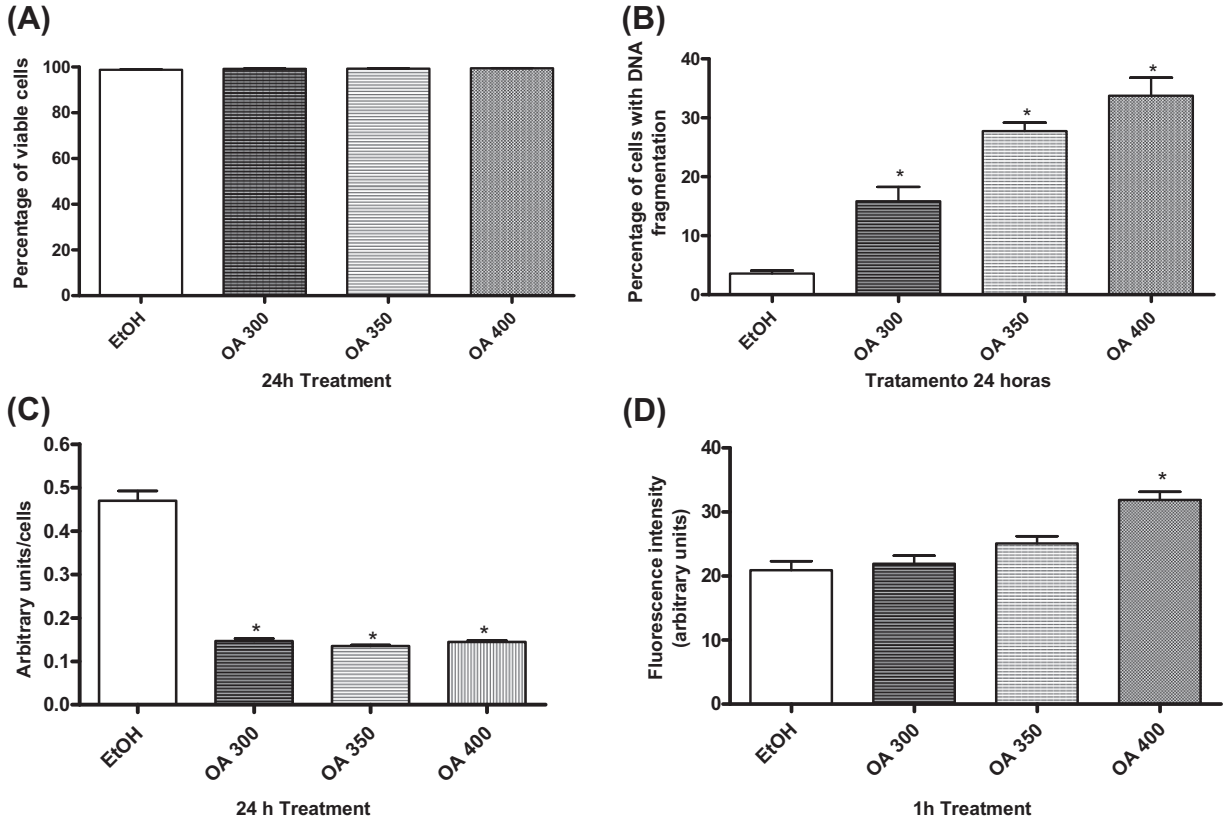

Fig. 3. ECV-304 endothelial cells incubated with oleic acid for 24 h. (A) Cell viability; (B) DNA fragmentation: ECV-304 endothelial cells were incubated with fatty acids for 24 h and analyzed by flow cytometry. The fluorescence was measured in FL2 channel (585/42 nm). (C) Oil red O staining: ECV-304 endothelial cells were incubated with fatty acids for 24 h and then were washed, fixed and stained with oil red O. The cells were examined by light microscopy and by absorbance assay at 510 nm. (D) Fluorescence intensity relative to the content of reactive oxygen species: ECV-304 endothelial cells were incubated with fatty acids for 30 min and then with hydroethydine for 30 min. Subsequently, the cells were analyzed to determine the production of superoxide anion in a fluorescence microscope using 590/46 nm filter. The graph shows the values of production of ROS induced by fatty acid treatment subtracted of the values obtained with ethanol. Values are presented as mean ± SEM ($n = 9$). $^{*}p < 0.05$ compared to ethanol as indicated by ANOVA, $^{\#}p < 0.05$ compared to oleic acid as indicated by ANOVA. EtOH = ethanol; OA 300 = oleic acid at 300 μM; OA 350 = oleic acid at 350 μM; OA 400 = oleic acid at 400 μM.

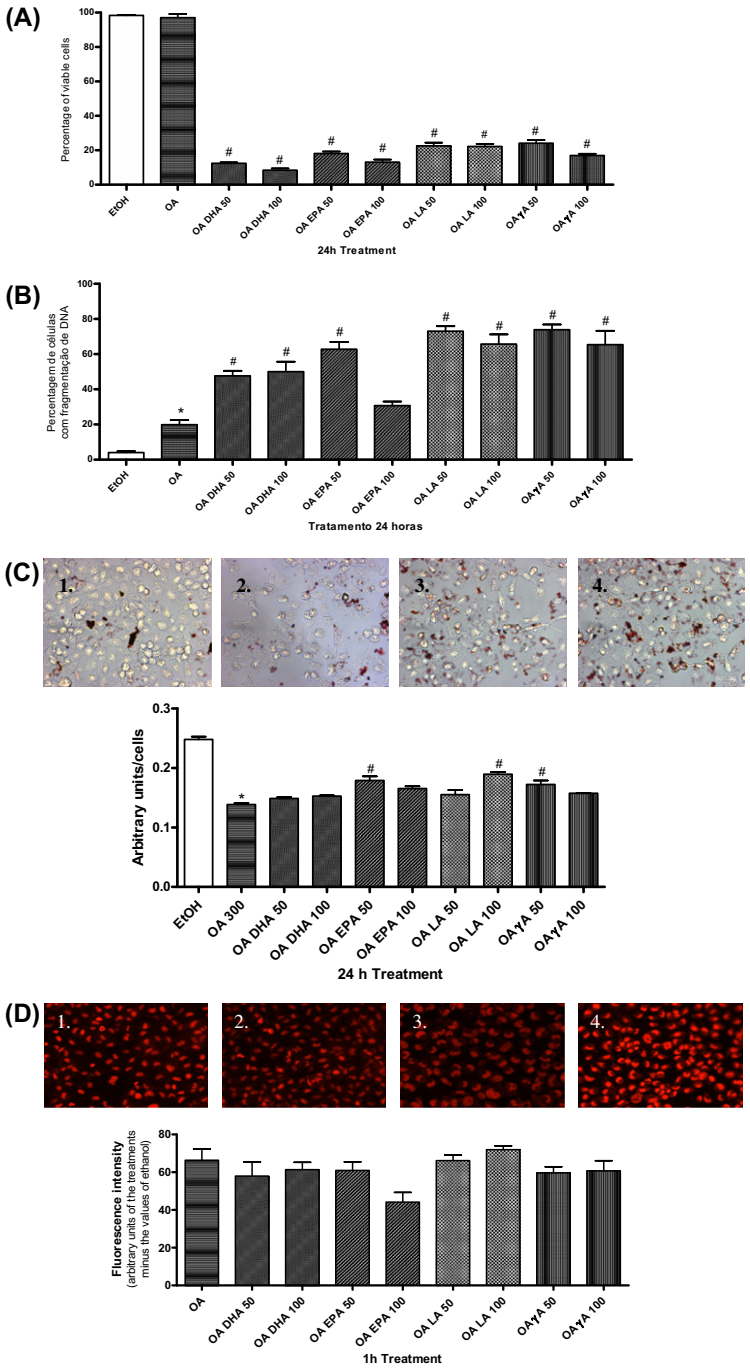

Fig. 4. ECV-304 endothelial cells incubated with oleic acid combined with ω-6 or ω-3 fatty acids for 24 h. (A) Cell viability; (B) DNA fragmentation: ECV-304 endothelial cells were incubated with fatty acids for 24 h and analyzed by flow cytometry. The fluorescence was measured in FL2 channel (585/42 nm). (C) Oil red O staining: ECV-304 endothelial cells were incubated with fatty acids for 24 h and then were washed, fixed and stained with oil red O. The cells were examined by light microscopy and by absorbance assay at 510 nm. (D) Fluorescence intensity relative to the content of reactive oxygen species: ECV-304 endothelial cells were incubated with fatty acids for 30 min and then with hydroethydine for 30 min. Subsequently, the cells were analyzed to determine the production of superoxide anion in a fluorescence microscope using 590/46 nm filter. (C) The photos illustrate the most significant results; 1. ethanol; 2. oleic acid at 300 μM; 3. oleic acid at 300 μM plus eicosapentaenoic acid at 50 μM; 4. oleic acid at 300 μM plus linoleic acid at 100 μM; (D) The photos illustrate the most significant results; 1. ethanol; 2. oleic acid at 300 μM; 3. oleic acid at 300 μM plus eicosapentaenoic acid at 100 μM; 4. oleic acid at 300 μM plus γ-linolenic acid at 50 μM; (D) The graphs show the results of fluorescence intensity of the treatments minus the values obtained with ethanol, expressed as mean ± SEM of three experiments performed in duplicate. $^{*}p < 0.05$ compared to ethanol as indicated by ANOVA, $^{\#}p < 0.05$ compared to oleic acid as indicated by ANOVA. EtOH = ethanol; OA = oleic acid always at 300 μM; DHA = docosaexaenoic acid; EPA = eicosapentaenoic acid; LA = linoleic acid; γA = γ-linolenic acid; all at 50 or 100 μM as indicated.

further by 3-fold for DHA; 4-fold for LA and γA, at 50 and 100 μM, and by 4-fold for EPA at 50 μM as compared to OA (Fig. 4B).

The treatment with OA at 300 μM decreased the lipids content by 56% compared to vehicle. The association of OA with PUFA (ω-3 and ω-6) increased the NL content compared to OA at 300 μM by: 30% and 25% for EPA and γA, respectively, both at 50 μM and 37% for LA at 100 μM (Fig. 4C). OA associated with ω-3 and ω-6 PUFA did not alter the ROS production compared to OA (Fig. 4D).

4. Discussion

4.1. SA associated with ω-3 and ω-6 PUFA

FFA are important mediators of endothelial dysfunction, atherosclerosis and cardiovascular disease (Azekoshi et al., 2010). In this study, SA increased the EC death and ROS production, without affecting NL content. ω-3 PUFA did not protect EC from death induced by SA but increased the lipids content and decreased the ROS production. In contrast, ω-6 PUFA reduced cell death induced by SA, increased lipids accumulation and decreased ROS content.

SA-induced cell death confirms the results obtained in previous studies (Artwohl et al., 2004; Rioux and Legrand, 2007. Artwohl et al., 2008 showed that SA causes apoptosis of various EC lines (HUVECs, HAECs, and EPCs HRECs). Saturated FA (stearic and palmitic acid) are the most abundant FFA in plasma (Hagenfeldt et al., 1972) and the major components of parenteral and enteral nutritional formulations, so the potential for adverse vascular effects initiated by saturated FA are cause for clinical concern. EC apoptosis plays an important role in endothelium dysfunction and directly affects blood thrombogenicity through the release of apoptotic microparticles into the bloodstream (Blann et al., 2009). ω-3 PUFA have important anti-inflammatory and anti-apoptotic properties (Massaro et al., 2008; Suphioglu et al., 2010). Artwohl et al. (2008) showed that low EPA levels (5–20 μM) inhibits SA-induced apoptosis in HUVEC, HAEC, EPC and HREC. In our study, EPA increased the percentage of viable cells without affecting DNA fragmentation induced by SA. However a marked decrease in the proportion of cells with death signs was found in the treatment with ω-6 PUFA and SA. No significant association between LA (ω-6 PUFA) intake (or tissues levels) and CHD risk (Esrey et al., 1996; Pietinen et al., 1997) and no consistent relations between stroke and LA intake (He et al., 2002; Sauvaget et al., 2004) have been found. Herein, ω-6 PUFA protected EC from death induced by SA.

SA did not affect EC NL content, but it does so in association of SA with ω-3 or ω-6 PUFA. Thus, PUFA, specially ω-6, may protect from SA-induced EC death by incorporating FA into NL (Cnop et al., 2001).

ROS have been implicated in the initiation and progression of atherosclerosis. ROS can oxidize lipoproteins, limit the vascular availability of antiatherosclerotic NO, and promote vascular expression of cytokines and adhesion molecules. Treatment of ECV-304 cells with SA for 30 min led to an increase of ROS. The combination of SA with ω-3 reduced ROS production but did not protect cells from SA-induced death. Our findings corroborate with those by Richard et al., 2008, showing that low ROS production by human aortic EC treated with ω-3 and ω-6 PUFA compared with saturated FA. In our study, the ω-6 PUFA led to a decrease in SA-induced ROS production. These findings contradict previous studies indicating that ω-6 PUFA have more potent effect on production of superoxide than the saturated FA (Schonfeld and Reiser, 2006, 2007). However, these authors did not investigate the combined effects of FA.

In general, ω-3 PUFA decreased ROS production, increased the content of NL but did not protect against EC death induced by SA. ω-6 PUFA inhibited SA-induced cell death, increased NL content and decreased ROS production. So, ω-6 PUFA have a greater protective effect than ω-3 PUFA on the deleterious effects caused by SA on EC.

4.2. OA associated with ω-3 and ω-6 PUFA

The consumption of the Mediterranean diet has been linked to greater longevity, improved quality of life and lower incidence of cardiovascular diseases (Carluccio et al., 2007). The Mediterranean diet is low in saturated FA and high in monounsaturated FA, particularly in OA. Pacheco et al. (2008) observed that a meal with a high monounsaturated to saturated FA ratio has a significant postprandial benefit on markers of endothelial dysfunction in healthy and hypertriglyceridemic subjects.

The results of OA on cell death confirm those of Krieglstein et al. (2008) and Reinbold et al. (2008). These authors also observed apoptosis in HUVEC cells by treatments with OA at concentrations of 200–400 μM.

This effect was pronounced in the treatment of OA at 300 μM with ω-3 and ω-6 PUFA for 24 h. The increase in cell death due to treatments with OA and PUFA was not due to the high load of FA since OA did not increase loss of membrane integrity even at 400 μM.

The diversion of FFA into NL may have cytoprotective effect. The NL content was decreased by OA at 300–400 μM. OA with ω-6 and ω-3 PUFA increased NL content compared to OA. These results did not prevent cell death in these treatments. Stentz and Kitabichi (2006) did not observe endothelial activation and increased ROS production in human aortic EC treated with OA at 50–500 μM. The same was found herein for OA alone or in association with ω-3 and ω-6 PUFA.

In summary, we demonstrated herein that the effect of a specific fatty acid (beneficial or deleterious) is substantially affected by combination with other fatty acids. ω-6 and ω-3 PUFA associated with OA increased cell death with no change in the OA effect on NL and ROS content. OA had low cytotoxic effect per se. However, the combination of OA with ω-3 or ω-6 PUFA, presented marked toxicity for ECV-304 EC. These results contribute to the understanding of the effects of circulating fatty acids on endothelial cell function and dysfunction.

Conflict of interest statement

There is no conflict of interest between the authors.

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