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Original article

Mediterranean-like mix of fatty acids induces cellular protection on lipid-overloaded hepatocytes from western diet fed mice

Lyssia Castellanos-Tapia[a,b], María Elizabeth Tejero-Barrera[a], Soraya Salas-Silva[b,c], Arturo Simoni-Nieves[b,c], Alejandro Escobedo-Calvario[b,c], Luis E. Gomez-Quiroz[c,*]

[a] Nutrigenomics Laboratory, National Institute of Genomic Medicine, Mexico City, Mexico
[b] Posgrado en Biologia Experimental, DCBS, Universidad Autonoma Metropolitana Unidad Iztapalapa, Mexico City, Mexico
[c] Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana Iztapalapa, Mexico City, Mexico

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ABSTRACT

Introduction and objective. Non-alcoholic fatty liver disease remains as one of the main liver disorders worldwide. It is widely accepted that is the kind of lipid, rather than the amount deposited in the cells that determines cell damage. Cholesterol and saturated free fatty acids are deleterious lipids when accumulated but, in contrast, there are some valuable lipids that could counteract those with harmful properties. Much of this knowledge arises from studies using a single fatty acid, but the effects of a combination of fatty acids, as obtained by diet has been poorly addressed. In the present work, we were focused to figure out the cellular effect of two different mixes of fatty acids, one with high proportion of saturated fatty acids, and another one with high proportion of unsaturated fatty acids (Mediterranean-like) in a cellular model of steatosis. Material and methods. Primary mouse hepatocytes from animals fed with a western diet (high fat and carbohydrates diet), were treated with both mixes of fatty acids for 24 h. Results. Our data clearly show that only the high unsaturated fatty acid mix induced a decrease in triglycerides (47.5%) and cholesterol (59%) content in steatotic hepatocytes mediating cellular protection associated to the decrement of ROS and oxidative damage. The mixture of high saturated fatty acids exhibited no effects, preserving high levels of cholesterol and triglycerides and oxidative damage. In conclusion, our results show that Mediterranean-like mix of fatty acids exerts cellular protection in steatosis by decreasing triglycerides, cholesterol, ROS content and oxidative damage.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) remains as one of the main liver disorders worldwide, with increasing prevalence every year [1]. It is associated with metabolic disorders, such as diabetes, dyslipidemia, metabolic syndrome, or obesity [2], but also to the consumption of fatty foods, particularly those rich in cholesterol, saturated free fatty acids (FFA) and carbohydrates such as western-style diet [3,4].

We and others have reported that is the type of lipid, rather than the amount that determines the severity of the disease [5–7]. In this context, high cholesterol overload in hepatocytes seems to be the main cellular noxious stimulus in NAFLD particularly because cholesterol targets mitochondria inducing oxidative stress, ATP decrement and susceptibility to cell death due to a second hit [5,7], such as alcohol toxicity [8], obstructive cholestasis [9], or deficiencies in signaling pathways, such as HGF/c-Met [10]; as a consequence oxidative stress is exacerbated increasing damage and, eventually, leading to carcinogenesis [6].

Diet could also be the counterbalance in NAFLD particularly due to the differential lipid content. It is recognized that Mediterranean diet is rich in monounsaturated and polyunsaturated fatty acids (MUFA and PUFA, respectively), which in addition present antioxidant properties, among others [11].

Diet is a determining factor that influences the concentration and composition of fatty acids in plasma and for instance, in the liver. Some studies have shown that certain fatty acids alone or in combination induce significant changes in liver physiology and

Abbreviations: NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; TG, Triglyceride; HMPFA, High mono and polyunsaturated fatty acids; HSFA, High proportions of saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; FFA, Free fatty acid; AST, Aspartate aminotransferase; ALT, Alanine transferase; ROS, Reactive Oxygen Species; TBARS, Thiobarbituric acid-reactive substances.

* Corresponding author.
E-mail address: legq@xanum.uam.mx (L.E. Gomez-Quiroz).

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pathophysiology. Hepatic cells treated with palmitic acid and n-3 PUFA increased the expression of genes related with beta oxidation, diminishing the toxic effects of the palmitic acid [12]. In primary rat hepatocytes stimulated with oleic acid in the presence of palmitic acid alleviated ER stress induced by palmitic acid [13].

There is evidence that high levels of palmitic, palmitoleic and myristic fatty acids in hepatic tissue from mice with steatosis is correlated with severity of NASH [14], similar findings have been reported in patients with NASH where high content of palmitic, myristic, palmitoleic, vaccenic and oleic acids were found in liver, this FFA mix was toxic in both human primary hepatocytes and in HepG2 cell line [15]. Furthermore, in terms of nutrition, dietary fatty acids come in complex combinations which can vary greatly in the content of SFA, MUFA or PUFA.

In the present work, we were focused to study the cellular effects of two different combinations of fatty acids, where the proportion of them was variable, to explore the differential response in lipid overloaded hepatocytes from mice fed with a western diet, as a mouse model of NAFLD.

2. Materials and methods

2.1. Experimental design

All animal procedures were carried out according to the guidelines of the NIH Guide for the Care and the Use of the Laboratory Animals and Univerisidad Autonoma Metropolitana Iztapalapa (UAM-I) animal facility guide. The research was approved by the ethical committee of the UAM-I. Twenty 8-10 weeks old BALB/c mice were randomly separated in two groups. The first one was fed with a western diet (ssniff EF R/M acc TD88137 mod, catalog # E15721-34, 10 mm pellets. ssniff Spezialdiaten GmbH, Soest, Germany) for 1 week. The second group was fed with control diet (ssniff EF R/M CD88137 Control, catalog #E15720-04, 10 mm pellets), the nutrient contents are the same that the western diet, except that the atherogenic components were removed. All animals received water and diet *ad libitum*.

2.2. Analysis of liver function

Serum activity of aspartate aminotransferase (AST), and alanine transferase (ALT) were determined by using automated analyzer for clinical chemistry (Spotchem EZ, Arkray USA Inc, Minneapolis, MN, USA) following manufacturerś instructions. Blood samples were obtained by coauthor S.S.S. in the perfusion procedure, as follows.

2.3. Primary mouse hepatocytes isolation, purification and culture

Hepatocytes were isolated from mice by the two-step collagenase perfusion technique, followed by isodensity purification in percoll gradient, as we previously described [16]. Briefly, mice were anesthetized with avertin, perfusion was performed in the mouse liver through the inferior vena cava via the right atrium, at this point blood was collected. The organ was perfused 5 min with Hank' balanced solution w/o calcium and magnesium containing 10 mM HEPES and 0.2 mM EGTA followed by 10–15 min perfusion with William's medium E containing 10 mM HEPES and 0.03% collagenase H (0.19 U/mg) (Boehringer Mannheim Biochemica).

Hepatocytes were seeded at 2.13×10^5 cells per cm^2 either in Lab-Tek chambered slides or 10-cm dishes (Nalge, Nunc) in the Ham's F-12/Dulbecco's modified Eagle's basal hepatocyte growth medium supplemented with ITS+ (Collaborative Research) (insulin 6.25 mg/ml, transferrin 6.25 mg/ml, selenious acid 6.25 ng/ml, bovine serum albumin 1.25 mg/ml, 2 mM glutamine, 30 mg/ml proline (Gibco Inc), 1 mg/ml galactose (Gibco), 18 mM HEPES, 1 mM sodium pyruvate (Gibco), 1.4×10^{-2} M sodium bicarbonate (Gibco) and 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT). After 4 h attachment, the medium was replaced by serum-free basal hepatocyte growth medium. The following day, cells were treated with different mix of FFA, according to Table 1. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), except when otherwise stated.

Table 1
Fatty acids composition of the two mixes.

FFA	HMPFA %	HSFA %	Sigma–Aldrich Catalog #
Palmitic	57	60	P0500
Myristic	0.5	1.5	M3128
Palmitoleic	1	3	P9417
Oleic	27	23	O1008
n-3 PUFA (EPA/DHA, 7/2)	14.5	6.5	E2011/D2534

Primary hepatocytes were treated with both FFA mixtures, the first one contained high proportions of saturated FFA (HSFA) and the second mixture contains high proportion of FFA monounsaturated (MUFA) and polyunsaturated (PUFA), and it is referred as (HMPFA) as Table 1 shows. All FFA were supplied by Merck and Co. (Kenllworth, NJ, USA). Treatments were carried out using 100 μM, final concentration, of each FFA mixture, for 24 h.

2.4. FFA mix formulation

Cell cultures (80% confluence) were under serum free media containing 1% antibiotics for 12 h at 37 °C, 5% CO_2. FFA mix formulation was based on previous report [17]. FFA (all >99% purity and purchased from Sigma-Aldrich, Saint Louis, MO, USA) mixes were prepared as follow: 100 mM stock of Palmitic acid and Myristic acid were dissolved in 100% ethanol at 70 °C, meanwhile oleic, palmitoleic acids and PUFA n-3: docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA); (7:2 EPA/DHA,) in 100% ethanol at 40 °C. Previously, albumin (fatty acids free, Sigma-Aldrich) was prepared in William medium (25 mg/mL), prewarmed at 40 °C until solve and filtered (0.45 μm). The conjugation of BSA-FFA was added to a prewarmed 25 mg/mL BSA solution and 2 mM of two mixes fatty acids overnight (40 °C). The conjugated mix was added to a pre-warmed media (40 °C) to achieve a final FFA mix concentration of 100 μM. Mix of FFA was based on the reported composition in human plasma of participants exposed to a diet with a high content of saturated fat [18] and persons living in the Mediterranean area [19]. The mixtures included same fatty acids with two different proportions of SFA, MUFA and PUFA n-3, as Table 1 depicts. The graphical experimental design is depicted in Supplementary Fig. S1.

2.5. Red oil staining

Neutral lipids cellular content was assayed by red oil O staining as previously published [20]. Briefly, cells were seed in chamber slides, after treatments, cells were washed with ice-cold PBS and then fixed with paraformaldehyde for one h. Then, cells were stained with 0.2% oil red O solution (Sigma-Aldrich, Inc) for 4 h. Cells were washed three times with isopropanol for 10 min. Nuclei were counterstained with hematoxylin. Microphotography was obtained in an Axioscope microscope (Carl Zeiss AG, Oberkochen, Germany)

2.6. Cell functionality by MTT

Cell functionality was addressed by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) test, using the

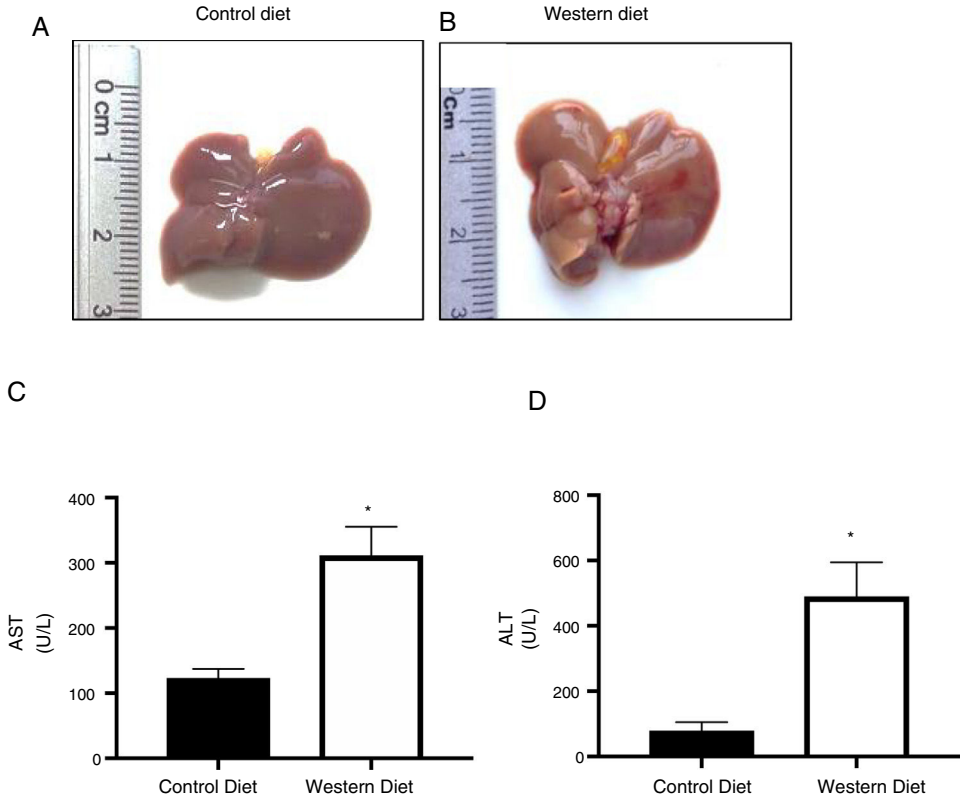

Fig. 1. Western diet induces liver dysfunction and steatosis. Macroscopic liver inspection A) Liver from mouse fed with control balanced diet; B) Liver from mouse fed with western diet for 7 days; C) Aspartate aminotransferase (AST) activity; D) Alanine aminotransferase (ALT) activity. The images are representative of at least four experimental animals. Each bar represents the mean ± SEM in at least four different mice, * $p < 0.01$ vs control diet.

Vybrant MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), following manufacturer's instructions.

2.7. Reactive oxygen species determination

Reactive oxygen species (ROS) cellular content was determined by dihydroethidium (DHE, 50 μM) fluorescence, for 15 min, in the dark, at room temperature as we previously reported [21]. DHE-derived fluorescence was recorded in a Multimodal reader (DTX 880, Beckman Coulter, Inc. Brea, CA, USA) at wavelength 520 nm for excitation and 570 nm for emission. Data is reported in arbitrary fluorescence units (AFU).

2.8. Lipid peroxidation

Lipid peroxidation was assayed by the production of thiobarbituric acid-reactive substances (TBARS) using spectrophotometry as described previously [21]

2.9. Protein content determination

Protein content was determined using the bicinchoninic acid (BCA), kit (Thermo Fisher Scientific), according to manufacturer's instructions.

3. Statistical Analysis

Data are presented as mean ± SEM of at least three independent experiments carried out by triplicate. Comparisons between groups were made using Student's *t* test, Mann Whitney and Tukey–Kramer test. GraphPad Prism 8 software for OSX was used to run analysis. Differences were considered significant at $p \leq 0.05$.

4. Results

We fed BALB/c mice with either western or control diet for seven days. As Fig. 1B shows, livers from animals under western diet exhibit the characteristic pale color of steatosis, comparing with control diet (Fig. 1A) even more, these animals presented high serum activity of AST and ALT, two well-accepted markers of liver damage (Fig. 1C and D), confirming liver injury due to high lipid diet consumption.

Due to the main objective of the present work was to figure out the impact of different combination of FFA in the lipid-overloaded hepatocyte, we proceeded to liver perfusion and hepatocytes isolation and culture (Supplementary Fig. S1).

To confirm the lipid overload in hepatocytes, neutral lipids were assessed by the oil red O staining, hepatocytes from animals under western diet showed increased lipid content in comparison with hepatocytes from control mice (Fig. 2A and B). To gain more confidence, we confirmed cellular lipid accumulation biochemically by measuring both triglycerides (TG) and cholesterol content. TG increased 2.65-fold (Fig. 2C) and cholesterol 4.98-fold (Fig. 2D) in western diet hepatocytes regarding control.

Results clearly show that western diet induces liver impairment and lipid overloaded hepatocytes, confirming that these cells represent a good model to study the cellular effects of differential FFA mixture in steatotic hepatocytes.

Cells from both groups of animals, were treated with 100 μM of HMPFA or HSFA mixes of FFA (Table 1) for 24 h. Interestingly, the treatment with HMPFA for 24 h remarkably decreased the lipid content (Fig. 3D) induced by western diet consumption (Fig. 3B), but no differences were observed in HSFA-treated cells (Fig. 3F). Control cells showed a slight increment in lipid content under the treatment with HMPFA, judged by oil red O staining, comparing

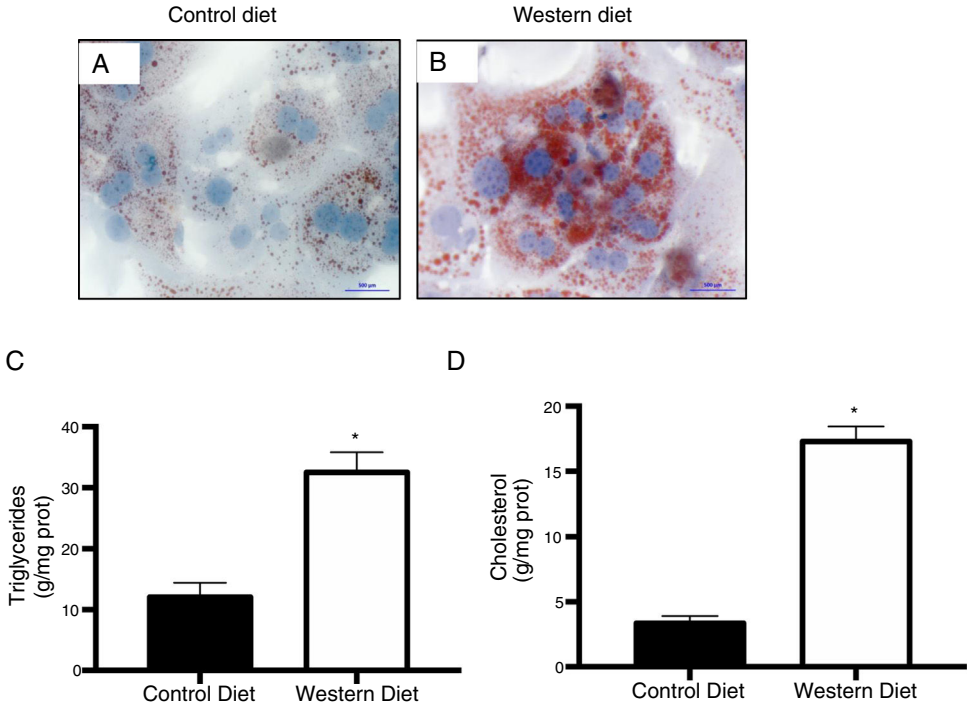

Fig. 2. Hepatocytes from mouse fed with western diet exhibits lipid overload. Neutral lipids content determined by oil red O staining. Primary hepatocytes from A) mouse fed with control balanced diet; B) mouse fed with western diet for 7 days; C) Liver triglyceride content; D) liver total cholesterol content. Data are normalized versus mg of cellular total protein. The images are representative of at least four animals. Reference bar 500 μm. Each bar represents the mean ± SEM in at least four different mice, *$p < 0.01$ vs control diet.

with not treated control cells (Fig. 3A), however these cells treated with HSFA observed a notable increment in lipid content (Fig. 3E). These results clearly show a favorable effect induced by the FFA Mediterranean-like mix treatment in steatotic hepatocytes.

To gain more confidence, we proceeded to biochemically measure both TG and cholesterol cellular content. Fig. 3G clearly shows that HMPFA treatment significantly reduces 2.1-fold the TG content in western hepatocytes, similar findings were found in cholesterol decreasing 1.7-fold regarding western hepatocytes (Fig. 3H), but HSFA mix did not observe any change, remaining TG and cholesterol values similar to those observed in western hepatocytes.

Cellular functionality, addressed by MTT assay, revealed a significant improvement in cell function in western hepatocytes treated with HMPFA mix comparing with not treated western cells (Fig. 4), while HSFA mix worsened cell function of western hepatocytes.

To gain mechanistic enlightenments, and due to oxidative stress is one of the main inducers of cell damage in steatosis as we previously published [6,7], we proceeded to measure the ROS content in cells under different treatments as previously reported [6]. Fig. 5A shows a remarkable increment in ROS in western cells compared with control hepatocytes, consistent with our previous findings. HMPFA mix remarkably reduced ROS production, while HSFA treatment did not induce significant changes in ROS content in western hepatocytes, even more, it increased the ROS in control hepatocytes comparing with not treated cells. These data were in agreement with lipid peroxidation, a well-accepted marker of oxidative damage, Fig. 5B shows that western hepatocytes increased 208.7% the TBARS regarding control cells, while HMPFA treatment decreased them 52% regarding western hepatocytes. Interestingly, HSFA increased TBARS in control cells, with no significant change in western hepatocytes, suggesting a kind of saturation in the oxidative damage.

5. Discussion

Recently, it has been published the last annual report to the nation on the status of cancer [22], this dossier leaves clear that liver malignancies are the most prevalent among all types of cancers. This is alarming. In part, the phenomenon could be related to the remarkable prevalence of NAFLD, not only in USA but worldwide [23], which seems to be increasing particularly by sedentary lifestyle and inadequate dietary behaviors. Although, it is well known that practically all liver diseases present a natural history starting with steatosis, this could also be as a consequence of the high consumption of fat and carbohydrates characterizing the typical western diet.

In the present work, we were aimed to address the cellular effects of different mixes of dietary FFA in lipid-overloaded primary mouse hepatocytes.

Although, many cancer-derived cell lines naturally present aberrant lipogenesis leading to lipid overload [24], the anomalous gene expression of these cells could mask potential interesting effects, particularly in metabolism, proliferation or survival. To address both, the effect of cellular lipid overload, and the impact of the treatment with specific lipids mixtures, we used a proper cellular model, with naturally occurring lipid overload. Standard commercial western diet (ssniff, E15721-34) provides high content of fat and carbohydrates (crude fat, 21.2%; cholesterol, 2.071%, and carbohydrates 33.2%) certainly resembling the western diet consumed in Mexico and developed countries such as USA [25].

Hepatocytes obtained from mice fed with western diet for one week, presented a clear lipid overload characterized by increased content of TG and free cholesterol (Fig. 2). Mice under western diet presented increased serum activity of AST and ALT and the characteristic pale color of the organ, indicating liver dysfunction due to the steatosis (Fig. 1).

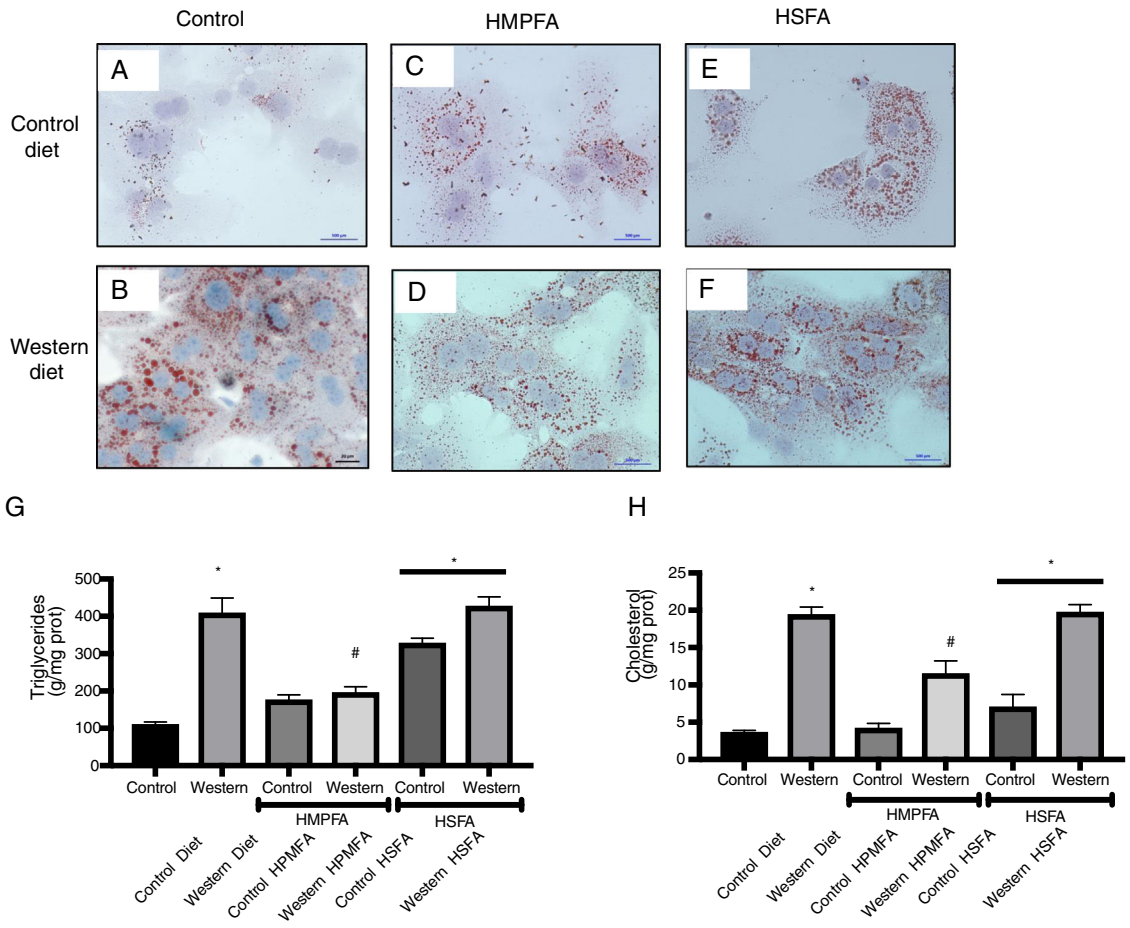

Fig. 3. Treatment with different lipid mixtures induces differential response in steatotic hepatocytes. Neutral lipids content determined by oil red O staining. Primary hepatocytes from mouse fed with control balanced control diet A), C) and E); or western diet B), D) and F) for 7 days. Cells were treated or not with high mono and polyunsaturated fatty acids (HMPFA) or high saturated fatty acids (HSFA) according Table 1, for 24 h. G) cellular triglycerides content and, H) total cellular cholesterol content. The images are representative of at least four animals. Reference bar 500 μm (200X original magnification). Each bar represents the mean ± SEM in at least four different mice. * $p < 0.05$ vs control cells, # $p < 0.05$ vs western cells.

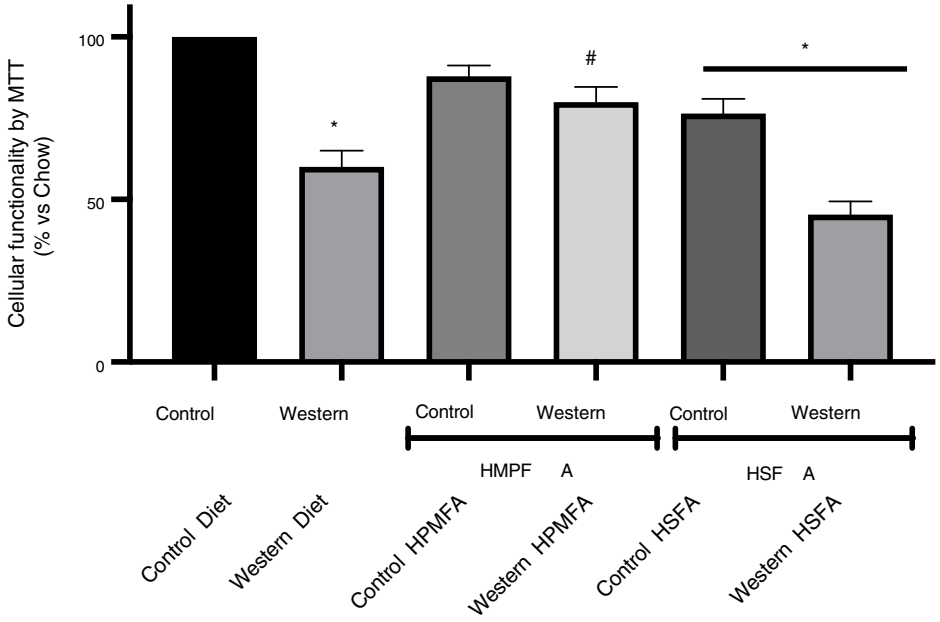

Fig. 4. Cellular functionality by MTT assay. Control and western cells were treated or not with high mono and polyunsaturated fatty acids (HMPFA) or high saturated fatty acids (HSFA) according Table 1, for 24 h. Each bar represents the mean ± SEM in at least four mice, *$p < 0.05$ vs control cells, # $p < 0.05$ vs western cells.

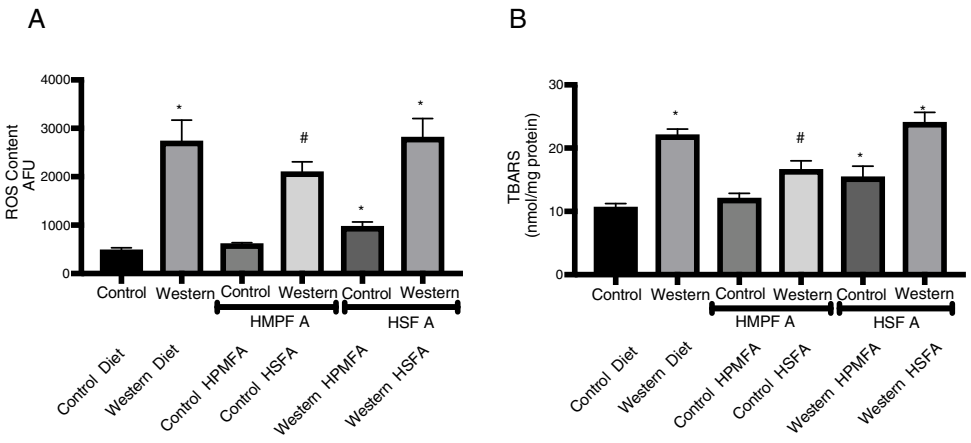

Fig. 5. Mediterranean-like mix of fatty acids protects lipid-overloaded hepatocytes from the oxidative damage. Control and western cells were treated or not with high mono and polyunsaturated fatty acids (HMPFA) or high saturated fatty acids (HSFA) according Table 1, for 24 h. (A) Reactive oxygen species (ROS) content determined by the superoxide radical probe dihydroethidium as reported in material and methods section; (B) Lipid peroxidation assayed by thiobarbituric acid reactive substances (TBARS). Each group represents mean ± SEM in at least three mice, *p < 0.05 vs control cells, # p < 0.05 vs western cells.

We and others have reported that is the kind of lipid rather than the amount of them that determines the grade of cellular damage [5,6,9], particularly the increment in hepatic cholesterol governs severity of liver diseases as we previously reported [8,9,26]. In our *in vitro* model, we show that cholesterol is significantly increased (5-fold) in hepatocytes. The excess of cholesterol in cells particularly targets mitochondria, inducing oxidative stress, mitochondria fission, decrement in ATP production and sensitization to damage [5,7]. Hepatocytes isolation by the two-step collagenase perfusion leads to the preservation of the steatotic phenotype, as we clearly show (Fig. 2). Cholesterol overload in addition, represents another challenge, persistent liver accumulation could induce apoptosis resistance [7] promoting tumorigenesis, as we recently reported [6].

There is no doubt regarding the problem of liver cholesterol accumulation. New alternatives directed to decrease noxious lipids are required, particularly nutritional ones.

It has been extensively published that the Mediterranean diet which is plant-centric, but moderate to low amounts of animal components, could play key role in chronic disease prevention [27–29]. Recently, it has been published that Mediterranean diet also provides beneficial effects in patients with NAFLD, diminishing parameters of severity such as lipid profile, glycemic index, serum liver enzymes activity, among others [30,31].

Focused exclusively in some the main types of FFA such as those find in plasma in persons who consume Mediterranean diet [19,32], we explored, at the cellular level, the effects induced by this FFA mix (HMPFA) on steatotic hepatocytes, comparing with a saturated FFA mix (HSFA). It is important to emphasize that both FFA mixtures are composed with the same lipids but with different content (Table 1).

It could be paradoxical, but our data clearly show that the presence of some lipids could influence the content of others. Fig. 3 depicts that HMPFA induces lipid content reduction in western hepatocytes, while HSFA seems to have no influence judged by oil red staining.

In addition to essential nutritional functions, FFA activate multiple signaling pathways, particularly those related to metabolism, by interacting with specific FFA receptors [33]. It is evident that HMPFA are activating changes in lipid metabolism or mobility, effect not observed with the saturated-rich mix. Although, both lipid combinations have the same fatty acids, the HMPFA has more n-3 PUFA. Some studies have shown that dietary supplementation with n-3 PUFA in obese mice under a high fat diet reduces hepatic lipid content, normalize TG with an improvement in the inflammatory response and decreasing oxidative stress, favoring insulin sensitivity, same effects have been observed in hepatic cells with lipid overload [34–36].

It is interesting that HMPFA exerted differential effect preferent controlling TG (Fig. 3G) rather than cholesterol (Fig. 3H), although cholesterol certainly decreased with the HMPFA treatment regarding untreated animal under western diet, cholesterol levels remain significantly increased when comparing with cells from control diet fed mice however, the decrement in cholesterol was related to an improvement in mitochondrial function judged by MTT test (Fig. 4).

It has been extensively published that cholesterol overload in hepatic cells is particularly relevant in mitochondrial functionality. Cholesterol accumulates in this organelle inducing oxidative stress and sensitization to a second insult [5,6,9].

Our data clearly show that, although the decrement of cholesterol induced by HMPFA treatment, it did not reach basal values, but it was good enough to decrease ROS content (Fig. 5A) and oxidative damage, judged by lipid peroxidation (Fig. 5B). Although, it has been observed that Mediterranean diet has the ability to decrease ROS by a polyphenol-dependent mechanism [37], the antioxidant properties elicited by mono or polyunsaturated FFA should be revisited, particularly in the context of NAFLD as recently published [11], and as our results clearly show.

Olive oil represents the main fat source in the Mediterranean diet, it is composed particularly of monounsaturated fatty acids (mainly oleic acid, 55-83%) and polyunsaturated fatty acids (4–20%) [11], in fact these FFA are excellent ROS scavengers due to the capacity of their double bonds to react with reactive substances, our data provide evidence that the HMPFA induced a decrement in ROS content and cellular lipid peroxidation (Fig. 5), mediated by the western diet.

PUFA, particularly n-3, have demonstrated to display protective effects in the liver, in fact, it is well known that enteral nutrition in hospitals is associated to deterioration of liver function, n-3 PUFA induced an improvement in the liver [38], the effect was associated to the downregulation of Cyp450 enzymes, this could represent an adaptive mechanism tending to improve oxidative stress phenomena.

Aside this remarkable protective effect induced by HMPFA, the HSFA mix did not exert any change in lipid-overloaded hepatocytes. As expected, treatment with HSFA in control hepatocytes increased cellular lipid overload by a significant increment of TG but not cholesterol. HSFA treatment resulted in a slight increment in ROS content and lipid peroxidation.

6. Conclusion

Our results show that combination of increased proportions of MUFA and PUFA exert cellular protective effects by controlling the levels of TG and cholesterol and decreasing the ROS production and the oxidative damage in hepatocytes with lipid overload induced by the feeding of a western diet. Consumption of increased combination of MUFA and PUFA and low content of saturated FFA, induces a protective effect in steatotic hepatocytes, leading to the improvement of cellular function by decreasing oxidative stress. The ingestion HMPFA (or Mediterranean-like diet) could potentially be a good adjuvant treatment in fatty liver diseases. Although, the *in vitro* study is confined to the effects in the hepatocytes, validation in *in vivo* models and, even more in humans, would be of great interest to stablish dietary supportive intervention in the treatment of NAFLD.

Conflict of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aohep.2020.06.005.

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