Nonalcoholic Fatty Liver Disease and Vitamin E - A Promising Relationship ?

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Oxidative stress (OS) plays an important role in NAFLD molecular mechanism. Nanoencapsulation represents a novel strategy to enhance therapeutic potential of conventional drugs. Our study analyses the encapsulated vitamin E effect on lipid metabolism and oxidative stress biomarkers in NAFLD rats. Animals were divided into 3 groups : G1 - the normal diet group; G2- the high caloric diet group ; G3 - high-caloric diet group receiving PLGA-vit E, 50 mg / kg. Serum advanced human oxidative protein (AOPP), total antioxidant capacity (TAC) and vitamin E were analysed using ELISA technique. Our results showed significant increase of G2 GPT, ALP, GGT, TG, glucose, TC and AOPP, versus G1 ($P < 0.05$) and a significant decrease of G2 serum TAC and vitamin E versus G1 results ($p = 0.01$ and 0.01). Vitamin E nanoparticles (G3) caused a significant increase of TAC and significant decrease of serum AOPP, versus G2 ($p < 0.01$). Results showed a significant reduction of GPT, GGT, ALP, TG and total cholesterol ($p < 0.05$) in G3 versus G2. PLGA nanoparticles should be considered an attractive and promising alternative to improve the bioavailability and biological activity of vitaminE.

Keywords: nanoparticles, vitamin E, oxidative stress, liver disease

Nonalcoholic fatty liver disease (NAFLD) become a worldwide health problem because it may progress to end-stage liver disease [1].

By the year 2015, the NAFLD global incidence of was estimated to 25.24%, suggesting that more than 1 billion people have NAFLD worldwide [2].

The higher prevalence of NAFLD is correlated with higher economic status [3]. Recent studies have indicated that excess calorie intake and more comfortable lifestyles with less exercise could be important players on the development of fatty liver disease scene [4].

NAFLD, considered the hepatic manifestation of metabolic syndrome, is associated with obesity, insulin resistance, hypertension, and dyslipidemia [5,6].

Singh and colab. illustrated that oxidative stress (OS) plays an important role in the molecular mechanism of NAFLD [7]. There are recent studies regarding the effects of antioxidants in NAFDL context leading to controversial results [8-10].

Currently there are no effective therapies for NAFLD, consequently more and more research efforts are focused on understanding its molecular mechanism in order to identify novel therapeutic strategies. In this sense, this study analyses the effect of encapsulated vitamin E on lipid metabolism and oxidative stress biomarkers in rats with NAFLD. As far as we know, there are no reports regarding the effect of encapsulated vitamin E in PLGA nanoparticles in the context of NAFLD.

Nanoencapsulation represents a novel strategy to enhance the pharmacokinetic and therapeutic potential of conventional drugs.

The low toxicity, controlled release and high bioavailability of biodegradable nanoparticles triggered their frequent use as drug delivery systems [11]. Poly(D,L-lactic-co-glycolic acid) (PLGA) is one of the most successful biodegradable polymers used for nanoparticle based medical treatments. In the human body PLGA is hydrolysed to glycolic acid, a non-toxic biodegradable metabolite [12]. Along with approval for use in humans by the US Food and Drug Administration, this polymer is a good candidate for preparation of drug delivery systems for diseases like cancer, schistosomiasis and malaria [11, 13-15].

Experimental part

Animal model

The rats were obtained from Animal Facility of Carol Davila University of Medicine and Pharmacy, Bucharest, Romania and the experimental procedures were carried out under Convention 86/609/E.E.C. from November 24, 1986, for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes.

The experiments began after acclimating the animals for 7 days under constant conditions of temperature (22 æ%C), humidity (55%), and light (12 h cycle dark/light) in polycarbonate cages. The animals were randomly divided into 3 groups and fed the normal or experimental diets for 5 weeks as follows: (1) G1 - the normal diet group (n = 10); (2) G2- the high caloric (high-fat and high - carbohydrates) diet group (n = 10); (3) G3 - the high-caloric diet group receiving in the morning a dose of PLGA-vit E, 50 mg / kg body administered by gavage (n = 10). Rats in G2, fed for 3 weeks a high-fat and high - carbohydrates diet developed NAFLD. The animals were allowed free access to food and water for five weeks. Food intake was measured daily, and the rats were weighed twice per week. At the end of the experiment period, rats were sacrificed after 12 h of fasting.

Vitamin E-loaded nanocapsules were prepared as described previously by .Miricescu D and colab [16].

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Biochemical Analysis

1. Whole blood samples were centrifuged and isolated serum was used for analysis of glucose, triglyceride (TG), total-cholesterol (TC), creatinine, ureea, cystatine C, glutamate pyruvate aminotransferse (ALT), alkaline phosphatase (ALP), GGT (gamma-glutamyl transferase) using analysing kits from Biosystems (Spain) on an automatic biochemistry analyser A25 -Biosystems (Spain).

2. Serum advanced human oxidative protein (AOPP) was also analysed using ELISA method. On this purpose we have used an analysing kit privided by RND – Germany. The microtiter plate is pre-coated with an antibody specific to AOPP. Standards and samples are added to the microtiter plate wells with a biotin-conjugated polyclonal antibody specific for AOPP and Avidin conjugated to Horseradish Peroxidase (HRP) are added to each microplate well and incubated. The TMB substrate solution is added to each well. Only the wells that contain AOPP, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is stopped by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm.

3. Fo*r vitamin E* measurements we have used an analysing kit privided by Biocompare - USA. This ELISA kit applies the competitive enzyme immunoassay technique utilizing a monoclonal antibody for the target antigen and a target antigen HRP conjugate. The assay sample and buffer are incubated together with target antigen HRP conjugate in pre-coated plate for one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex.

Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of color is inversely proportional to the target antigen concentration since the target antigen from samples and target antigen HRP conjugate compete for the antibody binding site. Since the number of sites is limited, as more sites are occupied by the target antigen from the sample, fewer sites are left to bind the conjugate. A standard curve is plotted relating the intensity of the color (O.D.) to the concentration of standards. The target antigen concentration in each sample is interpolated from standard curve.

4. Total antioxidant capacity assay (TAC) sau (Total Antioxidant Status-TAS, Randox, Crumlin, UK activity was performed using an analysis kit), on a semiautomatic biochemistry analyzer. The method is based on the ability of antioxidant molecules to quench the long-lived ABTSæ%+, a blue -green chromophore with characteristic absorption at 734 nm, in comparison to that of Trolox, a water-soluble vitamin E analogue.

Statistical Analysis

Statistical analysis was performed using *Student test* to compare and correlate clinical parameters with biochemical biomarkers. Statistical significance was set at a p-value of <0.05.

Results and discussions

In this study, we have used a diet-induced NAFLD rat model to investigate the influence of vitamin E encapsulated in PLGA nanoparticles on lipid metabolism and OS biomarkers.

Our study results are presented in table 1 and figures 1, 2 and 3.

Serum Parameters	G1 Control group	G2 NAFDL rats group	G3 NAFDL rats treated with vitamin E nanoparticles Group
Glucose mg/dL	78±38.52	118 ±11.34	98±11.34
Total Cholesterol mg/dL	106±8.2	163±4.95	134±2.98
TAG mg/dL	66±38.52	189 ±21.89	158±18.50
ALT U/L	35±6.52	77±7.2	58±5.1
GGT U/L	7.8±1.52	15.3±1.81	9.5±0.81
ALP U/L	210±8.52	276±12.5	206±7.9
Cystatine C mg/dL	0.172±0.09	0.177±0.01	0.173±0.08
Creatinine mg/dL	0.34±0.052	0.33±0.03	0.38±0.03
Ureea mg/dL	14.55±3.52	13.66±4.23	21.33±4.23
AOPP ng/mL	12.1±0.9	37.6±0.9	21.9±0.6
TAC mmol/L	79±10.88	63±9.2	86±12.89
Vit E mmol/L	68±8.52	49±11.87	100±11.87

Table 1
AVERAGE CONCENTRATION OF SERUM PARAMETERS FOR G1, G2 AND G3 GROUPS OF RATS

Our results showed that in G2 GPT, ALP, GGT, TG, glucose and total cholesterol were significantly higher compared with G1 (P- 0.05, 0.001, 0.001, 0.05, 0.001 and 0.05 respectively) (table 1 and figures 1 and 2), showing that rats in G2 have developed NAFDL.

G2 serum TAC and vitamin E levels were significantly reduced compared with G1 results (p= 0.01 and 0.01 respectivelly). In the mean time, we have noticed that G2 serum levels of AOPP were significantly incresed (p= 0.01) compared with G1 results (table 1, fig. 3). All these data clearly illustrated an incresed oxidative stress after administration of high fat diet for 3 weeks (fig. 3).

Comparing G1 and G3 results, it can be concluded that vitamin E nanoparticles administration had no significant effect on serum renal function parameters (creatinine, ureea and cystatine) and hepatocellular damage markers (ALT, GGT and ALP) (p > 0.5), (table 1, figs. 1 and 2).

Administration of encapsulated vitamin E in PLGA nanoparticles (G3) caused a significant increase of serum antioxidant biomarkers (GSH, TAC), *versus* G2 results (p< 0.01). We have also noticed that vitamin E nanoparticles administration in G3 significantly reduced serum AOPP, compared with the NAFLD group (G2) (p<0.01), (table 1, fig. 3).

Our study results also showed a significant reduction of serum levels for GPT, GGT, ALP, TG and total cholesterol (P – 0.01, 0.05, 0.01, 0.01 and 0.05 respectively) in G3 *versus* G2 results (table 1, figs. 1 and 2).

A lot of laboratory and clinical studies revealed that NAFLD is a complex metabolic issue. More and more recent efforts has been focused on identifying and novel potential targets in order to develop new therapeutical strategies for NAFLD, during its progression.

The aim of our study was to evaluate the effect of encapsulated vitamin E on serum lipid metabolism and oxidative stress biomarkers in rats with NAFLD.

NAFLD is considered one of the most frequent chronic liver disease worldwide due to obesity, induced by available high-energy food and sedentary lifestyle in modern society [17]. This is the reason why this metabolic disorder is gaining more and more attention in medical world [17, 18].

NAFLD pathogenesis is still very complicated and involves lipid accumulation, insulin resistance, inflammation, and fibrogenesis and can be closely

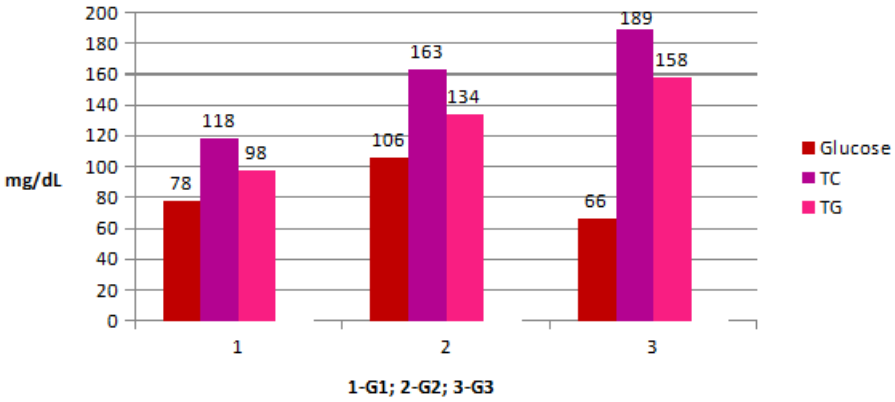

Fig.1. Average values of serum glucose, total cholesterol and triglycerides concentrations in G1 (control group - normal diet normal diet fed rats), G2 (rats with NAFLD) and G3 (NAFDL rats treated with vitamin E nanoparticles group), respectively

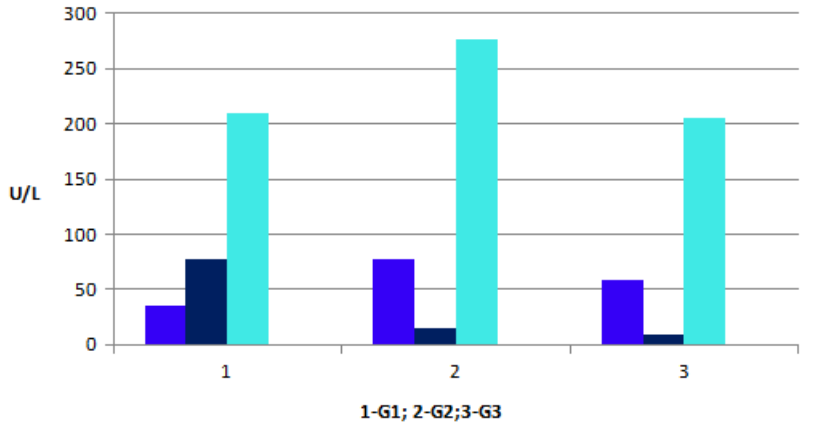

Fig. 2. Average values of serum ALT, GGT, ALP levels in G1 (control group - normal diet normal diet fed rats), G2 (rats with NAFLD) and G3 (NAFDL rats treated with vitamin E nanoparticles group), respectively

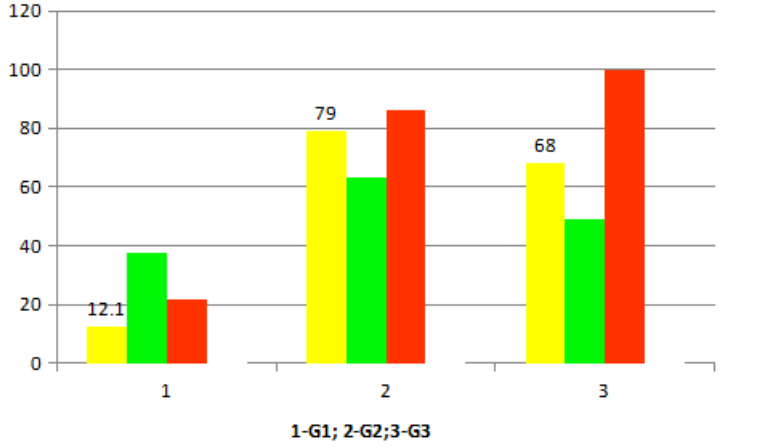

Fig. 3. Average values of serum AOPP, TAC and vitamin E concentrations in G1 (control group - normal diet normal diet fed rats), G2 (rats with NAFLD) and G3 (NAFDL rats treated with vitamin E nanoparticles group), respectively

associated with complications like obesity, hepatic steatosis, liver fibrosis and diabetes [19,20].

In our study serum lipid profile was assessed by measuring the changes in serum levels of total cholesterol and TG (as markers for lipid metabolism alteration). Our results showed that G2 TG and total cholesterol were significantly higher compared with G1 (p- 0.05 and 0.05 respectively) (table 1, fig. 1). Experimental data also ilustrated a significantly increased ($p < 0.05$) serum glucose level in G2 group *versus* G1. Liver functions were assessed by measuring serum levels of GPT (a specific marker for hepatic parenchymal injury), ALP (marker of the relative degree of hepatocellular damage and of obstruction, intrahepatic or extrahepatic) and GGT (marker for cholestasis and GSH status). Our data revealed that G2 GPT, ALP and GGT were significantly higher compared with G1 (p- 0.05, 0.001 and 0.05 respectively) (table 1, fig. 2). All these presented data showed that the G2 rats have developed NAFLD.

NAFLD hallmark molecular events are: 1. triglyceride accumulation in hepatocytes as a result of imbalanced lipid input and output; 2. increase in free fatty acids (FFAs) uptake from circulation due to increased lipolysis in adipose tissue and/or from the diet (as chylomicrons; 3. increase in glucose and insulin levels in response to carbohydrate intake t promoting de novo lipogenesis; 4. reduction of fatty acids mitochondrial oxidation; 5. decrease in hepatic triglyceride secretion by packaging with ApoB into VLDLs [21-24].

As shown in figure 3, G2 TAC and vitamin E levels were significantly reduced compared with G1 results (p- 0.01 and 0.01 respectivelly). G2 serum levels of AOPP were significantly incresed ($p = 0.01$) compared *versus* G1 results. All these data sugested an incresed oxidative stress in the high fat diet fed rats (G2).

Recent studies are showing that obese pacients have greater risks to develop nonalcoholic fatty liver disease (NAFLD) compared with general population [25]. Elevated serum levels of AST, ALT and ALP reflect liver cells injury caused by hepatic steatosis [25]. A high caloric diet (characterised by excessive intake of glucose and fat) causes hepatic accumulation of triglycerides, cholesterol and free fatty acids, leading to hepatic steatosis [26-28].

Total cholesterol and free fatty acids accumulation in liver cause an intens oxidative stress by increasing ROS production [29]. Cholesterol and FFAs, especially when are accumulated in mito-chondria, play the role of *aggressive* lipids which trigger TNFα-mediated liver cells damage and reactive oxygen species (ROS) formation [21,22]. In other words, FFAs cause lipotoxicity by exacerbating the ROS release, which, in turn, causes inflammation, apoptosis, and thus, the progression to NAFLD and fibrogenesis [22]. Consequently, these ROS will trigger lipid peroxidation reactions and malondialdehyde formation and induceing TNF-alpha -regulated liver cells damage [22,30]. β-oxidation of FA within peroxisomes and ω-oxidation within the ER are upregulated in NAFLD and contribute to lipotoxicity and ROS formation [31,32]. This might be secondary to inhibition of mitochondrial β-oxidation due to an accumulation of malonyl-CoA . In fact, recent studies indicate that activation of mitochondrial FA oxidation protects from steatosis and insulin resistence (IR) [31,32].

TNF-alpha -induced inflammatory reactions targeted against products of an abnormal lipid metabolism is considered the main cause of NAFLD pathology [21]. Hepatic inflammation characteristic for steatohepatitis inhance the oxidative stress and stimulate the mitigen-activated protein kinase pathway and the nuclear factor -kB , causing finally insulin resistance [33]. During the hepatic inflammation process Kupffer cells are recruited, macrophages M1 polarizes and the hepatic stellate cells are activated, finally induceing liver fibrosis [33] and accentuating the oxidative stress, which on its turn, will increase the inflamatory reaction.

All these findings sustain our experimental data indicating the existance of an important OS in NAFLD rats (G2) compared with group G1. This OS is illustrated by the significant reduction of serum G2 TAC and vitamin E levels (p- 0.01 and 0.01 respectivelly), (table 1, fig. 3) and by the incresed G2 AOPP serum level ($p = 0.01$), *versus* G1 (table 1, fig. 3).

OS have many effects on antioxidant defense mechanisms. It has been showen that overproduced ROS can directly deplete antioxidant molecules such as glutathione (GSH) and inhibit the activities of antioxidant enzymes such as superoxide dismutase (SOD), which could explain our results concerning serum TAC (table 1, fig. 3) [34].

Oxidative stress, the imbalance between free radical formation and its scavenging, has a crucial role in pathogenesis and progresion of NAFLD [35].

Reduction of oxidative stress represents an important potential therapeutic target for NAFLD patients. Recent attempts in finding efficient NAFLD therapy have focused on micronutrient antioxidants, that may counteract ROS accumulation and, finally, ameliorate the metabolic imbalance characteristic to this disease [36].

Micronutrient antioxidants, like vitamins and carotenoids, are mainly found in fruits and vegetables and are efficient protectors against OS instalation [37]. There are studies revealing that low antioxidant levels in the serum of patients with chronic liver diseases [36].

This is the reason why we have administered *encapsulated vitamin E t*o rats with diet-induced NAFDL in an attempt to observe this antioxidant vitamin's effect on the serum oxidative status and lipid metabolism markers. Vitamin E was encapsulated in PLGA nanoparticles because they have minimal systemic toxicity [38,39]. We have administrated the vitamin encapsulated in PLGA nanoparticules in order to improve its bioavailability and being aware of its light, heat and oxygen sensitivity.

Vitamin E is known as one of the most powerful antioxidants in nature [40-42].

Administration of encapsulated vitamin E in PLGA nanoparticles (G3) induced a significant increase of serum antioxidant biomarkers (GSH, TAC), *versus* G2 results ($p < 0.01$), (table 1, fig. 3). We have also noticed that vitamin E nanoparticles administration in G3 significantly reduced serum AOPP, compared with the high lipid diet group (G2) ($p < 0.01$), (table 1, fig. 3).

Our results illustrated that the administration of the encapsulated vitamin E reduced significantly the OS in the NAFLD rats group. In the circumstances of our study, vitamin E acted as an effective antioxidant. The antioxidant capacity is sustained by the hydroxyl group from the aromatic ring of tocochromanols, which donates one hydrogen atom to neutralize free radicals or ROS [43,44].

Interestingly, our experimental data also revealed a significant reduction of serum levels for GPT, GGT, ALP, TG and total cholesterol (p– 0.01, 0.05, 0.01, 0.01 and 0.05 respectively) in G3 *versus* G2 results (table 1, figs. 1 and 2). These findings suggest that vitamin E nanoparticles had a protective effect on the liver function of NAFDL rat models (G3) and, also, corected the lipidic profile.

Similar results were obtained by Sanyal et all and Lavine et all. [10,45,46]. These findings could be explained by the

fact that vitamin E biological activity is not limited to antioxidant properties. In fact, vitamin E plays important roles in inflammatory response, gene expression, cellular signaling, and cell proliferation regulation. This vitamin is involved in regulating specific genes expression concerning not only OS, but also involved in inflammatory pathways control and cholesterol homeostasis [47,48]. These genes encode for proteins involved in:

- inflammation pathways and cell adhesion (such as integrin, interleukin (IL)-1b, IL-2, IL-4, and transforming growth factor (TGF-β)
- in extracellular matrix formation and degradation (tropomyosin, glycoprotein IIb, collagen A1, matrix metalloproteinase (MMP-1, MMP-19 and connective tissue growth factor)
- cell cycle regulation (cyclin D1, cyclin E1, and p27)
- lipid metabolism (Cytochrome P450 3A4 and HMG-CoA reductase) [47,48].

On the other hand, but important to mention, there are recent studies underscoring that vitamin E can alternatively become under certain circumstances a pro-oxidant molecule. This circumstances could include the absence of co-antioxidants like vitamin C and a constant low-level flux of initiator free radicals [49].

Our study result and many other recent studies ilustrated how antioxidant therapy especially with vitamin E, reduces oxidative stress, inflammation, extracellular matrix remodeling and insulin resistance [10,45,46]. However, there are clinical studies using antioxidant therapy with vitamin E in oxidative stress-related diseases treatment which have shown contradictory results [50-53]. Stone and colab. have shown that vitamin E supplementation at doses higher than 400 UI/day may increase the risk of all-cause death [51].

Quite recent studies using cellular models revealed the pivotal role of ROS as second messengers in many molecular events, some of them active during the development of obesity and NAFLD. For example, H_2O_2 formation is necessary for adipocytes differentiation and adipogenesis, in a process that can be inhibited by antioxidant supplementation [54; 55]. In addition, ROS may also play an important role in the insulin signaling pathway [56].

Finally, all these data highlight the vital importance of keeping antioxidant molecules concentration within a physiological range in order to maintain metabolic homeostasis [56].

Conclusions

More and more attention has been focused on NAFLD, which could become one of the most severe chronic liver disease worldwide in the next decades. NAFLD pathogenetic molecular mechanisms are very complex being associated with metabolic complications, like hepatic fibrosis and type 2 diabetes. Consequently, novel preventive and therapeutic strategies are seriously required. These new strategies should include micronutrient antioxidants that resist oxidative stress and can normalize cellular redox status. Acquiring physical exercise habits together with controlled dietary supplements (including these micronutrients), could be a promising NAFLD management. For instance, the Mediterranean diet, containing silymarin phytosome complex and vitamin E, should be a starting point.

PLGA nanoparticles should be considered an attractive and promising alternative to improve the bioavailability and biological activity of liposoluble compounds such as vitamin E.

However, there are still necessary further studies in order to clarify NAFLD molecular mechanism, pathophysiology and micronutrients promising role in prevention and treatment of this disease.

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