

Effect of Essential Phospholipids and Statin on Non Alcoholic Fatty Liver Disease in Rats Induced Periodontitis

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Abstract: NAFLD is the most common form of chronic liver disease, it is the hepatic manifestation of metabolic syndrome. Periodontal disease may helpfully increase the risk of progression from NAFL to NASH. Effective strategies are needed to treat NAFLD and prevent progression to NASH so we conducted this study to investigate the efficacy of EPL and statin on biochemical parameters and histo-pathological finding in rats with NAFLD and periodontitis. Methods: 35 Male rats were divided into 5 groups, group 1: control group, group 2: NAFL, group 3: NAFL with periodontitis, group 4: rats with NAFL and periodontitis received EPL and group 5: rats with NAFL and periodontitis receiving statin after 30 days of treatment blood sample and mandibles were collected for biochemical analysis and histo-pathological findings. Result : group treated with statin significantly elevated (AST, ALT and γ GGT) and lipid profile while treatment with EPL significantly reduced liver enzymes and lipid profile parameters, both EPL and Statin reduce OPG level and improved bone resorption which confirmed by histological findings. Conclusion: We confirm the efficacy of EPL in accelerating the improvement of biochemical and pathological findings, such as liver function enzymes, lipid profile and bone resorption in rats with NAFLD and periodontitis.

Keywords: Biochemical Parameters, Essential Phospholipids, Non Alcoholic Fatty Liver, Periodontitis, Statin.

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I. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a medical condition in which excess fat accumulates in the patient liver without a history of alcohol consumption [1]. NAFL includes a variety of histo-pathological findings as simple fatty liver, non-alcoholic steatohepatitis (NASH) includes fibrosis and ultimately cirrhosis, which may progress to hepatocellular carcinoma [2]. NAFLD is considered as one of the hepatic manifestation of metabolic syndrome that is closely associated with multiple factors such as obesity, hyperlipidemia and type 2 diabetes mellitus. However, other risk factors for the development of NAFLD are unclear [3]. the pathogenesis of NAFLD is not completely understood, and treatment of the disease has not been fully defined. Periodontal diseases refer to the inflammatory processes that occur in the tissues surrounding the teeth in response to bacterial accumulations, they are the major cause of tooth loss in adults, and they can have a disturbing impact on oral function and appearance. Periodontitis is a complex disease, in which disease expression involves various interactions of the biofilm with the host immune- inflammatory response and subsequent alterations in bone and connective tissue homeostasis [4]. Alveolar bone destruction, a feature of periodontitis progression, is mediated by the host immune and inflammatory response to microbial challenge. Increased osteoclast activity without a corresponding increase in bone formation is a main characteristic of inflammatory bone loss in periodontitis. Some reports suggest that infection with *P. gingivalis* is associated with several systemic diseases, including cardiovascular diseases, preterm low birth weight, rheumatoid arthritis, and diabetes mellitus (DM) [5]. the development of systemic diseases receiving increasing attention recently Therefore, we conducted this study to investigate the effect of both essential phospholipids and statin on biochemical parameters and histo-pathological findings of rats with NAFLD and Periodontal diseases.

Statins are group of lipid lowering medications which show hypocholesterolaemic effect by inhibiting production of cholesterol in the liver. Statins are referred to as hydroxy-methylglutaryl-coenzyme A reductase inhibitors (HMG-CoA reductase) which plays a central role in the production of cholesterol [6]. Administration of statins are associated with many side effect as muscle pain, muscle damage, increased risk of diabetes mellitus [7]. Essential phospholipids (EPL) are highly-purified phosphatidylcholine fractions containing linoleic acid and other unsaturated fatty acids. Polyene phosphatidylcholine (PPC) is a major active ingredient in essential phospholipids [8]. It has a high bioavailability and affinity for cellular and subcellular membranes, and helps to maintain membrane fluidity and function. The nontoxic EPL have been already widely accepted to be effective in various liver diseases [9,10]. EPL shows antioxidant, anti-inflammatory, antifibrotic,

apoptosis-modulating, regenerative, membrane-repairing and -protective, cell signaling and receptor-influencing, and lipid-regulating effects [11].

II. Material And Methods

2.1 Animals, Diet and Treatment

Thirty five local Wistar male rats weighing approximately 100-120 grams obtained from animal house of Pharos university were used in the present study. All experiments were conducted in accordance with the guide for the care and use. Animals were housed 7 per cage and provided a commercial diet and tap water *ad libitum*. All animals were kept under observation for one week prior to study for acclimation in the laboratory environment. Rats were divided into control group (group I n=7) fed a standard diet and non alcoholic fatty liver (NAFL) group, (n= 28). fed high fat diet (HFD) for 14 weeks. The model was based on the model reported by (Fiorese, et al. 2008) [12]. The HFD consisted of commercial rat chow plus peanuts, milk chocolate, and sweet biscuit in a proportion of 3:2:2:1. All components of the high-fat diet were ground and blended.

2.2 Ligature-Induced Periodontitis

21 rats induced periodontitis, rats were first anesthetized with an intraperitoneal injection of ketamine and xylazine (90 and 15 mg/kg, respectively). A cotton ligature (4/0) was placed around the cervixes of both sides (right and left) of mandibular first molars and maxillary second molars in each animal (four ligatures/animal). The ligature was knotted on the vestibular side, so that it remained sub gingival on the palatal side. Sham-operated rats had the ligature removed immediately after the procedure (Brito et al., 2013) [13].

2.3 Grouping of Animals

After 14 days, the ligatures were removed and the rats were divided into four groups, 7 rats in each group, rats grouped as follows:

Group II. NAFL rats without any treatment

Group III. NAFL rats induced Periodontitis

Group IV. NAFL and Periodontitis rats received statin (2 mg/kg body weight) via or gastric tube.

Group V: NAFL and Periodontitis rats treated with essential phospholipids (100 mg/kg body weight) via orogastric tube for 30 days. At end of treatment period, the fasted rats were anesthetized with thiopentone 40 mg/kg IP, and blood samples were collected from the inferior vena cava, in sterile tubes which used for biochemical analysis.

2.4 Biochemical Analysis

The levels of triglyceride (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alanine transaminase (ALT), and aspartate aminotransferase (AST), were measured in the serum by the enzymatic colorimetric method using commercial reagents assay kits (Thermo Fisher Scientific Inc., USA). Osteoprotegerin (OPG) levels were determined by enzyme-linked immunosorbent assay (Aviscera Bioscience Inc. USA).

2.5 Histological Examination

Their heads were decapitated and mandibles were dissected out and cut into two halves. All specimens were fixed in 10% buffered formalin. The specimens were de-mineralized then processed into paraffin sections to be stained with hematoxylin and eosin (H & E) by Trichrome [14].

2.6 Statistical Analysis of Data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. ANOVA was used to compare between the five studied groups for normally distributed quantitative variables. Kruskal Wallis test for abnormally distributed quantitative variables, to compare between more than two studied groups, and Post Hoc (Dunn's multiple comparisons test) for pairwise comparisons. Significance of the obtained results was judged at the 5% level.

III. Result

3.1 Biochemical analysis

liver enzymes increased significantly in statin treated group compared with control, NAFL or NAFL and periodontitis. AST, ALT and γ -Glutamyl transferase were significantly decreased in group treated with EPL than control or NAFL and periodontitis groups "Table 1".

Table (1):Comparison between the studied groups according to liver function test in serum of rats

| | Control (n= 7) | NAFL (n= 7) | NAFL +perio (n= 7) | Statin (n= 7) | EPL (n= 7) | p |
|------------|-------------------|----------------|--------------------------|-----------------------------|----------------------------|-------------|
| AST (U/L) | 17.26 ± 1.31 | 43.50 ± 3.26 | 53.57 ± 3.95 | 63.82 ± 4.48 ^{abc} | 46.20 ± 9.4 ^{ac} | <0.001 * |
| ALT (U/L) | 15.76 ± 0.38 | 32.31 ± 2.17 | 56.67 ± 5.56 | 71.71 ± 9.50 ^{abc} | 44.20 ± 6.86 ^{ac} | <0.001 * |
| γ-GT (U/L) | 13.29 ± 0.41 | 42.43 ± 2.24 | 58.73 ± 4.83 | 49.20 ± 4.75 ^a | 47.43 ± 3.02 ^{ac} | <0.001 * |

p: p value for Kruskal Wallis test, Sig. bet. grps was done using Post Hoc Test (Dunn's multiple comparisons test) , a: Significant with Control , b: Significant with NAFL, c: Significant with NAFL+Periogroup

3.2 Lipid Profile

Table 2 shows that groups treated with both statin and EPL significantly decreased TC ,TG, and LDL-c compared with NAFL or NAFL and periodontitis with the best result to EPL while HDL-c significantly elevated compared with NAFL and periodontitis group.

Table (2):Comparison between the studied groups according to lipid profile in serum of rats

| | Control (n= 7) | NAFL (n= 7) | NAFL + perio (n= 7) | Statin (n= 7) | EPL (n= 7) | p |
|--------------------|-------------------|-------------------|------------------------|--------------------------------|--------------------------------|---------|
| Total-C (mg/dl) | 86.06 ± 9.92 | 141.79 ± 11.53 | 142.03 ± 13.01 | 98.84 ± 9.04 ^{bc} | 89.26 ± 14.49 ^{bc} | <0.001* |
| TG (mg/dl) | 59.26 ± 4.09 | 84.84 ± 3.71 | 87.02 ± 1.00 | 74.01 ± 1.88 ^{abc} | 71.62 ± 0.97 ^{abc} | <0.001* |
| HDL-C (mg/dl) | 54.40 ± 4.55 | 29.43 ± 10.47 | 19.37 ± 1.55 | 29.23 ± 4.05 ^{ac} | 32.11 ± 2.66 ^{ac} | <0.001* |
| LDL-C (mg/dl) | 27.06 ± 1.35 | 69.71 ± 3.75 | 69.27 ±5.89 | 52.53 ± 2.53 ^{abc} | 51.30 ± 4.10 ^{abc} | <0.001* |

p: p value for F test (ANOVA) for comparing between the different studied group

p: p value for Kruskal Wallis test, Sig. bet. grps was done using Post Hoc Test (Dunn's multiple comparisons test) , a: Significant with Control, b: Significant with NAFL, c: Significant with NAFL+Perio group.

3.3 Changes in circulating OPG

As shown in "Table 3" circulating OPG levels significantly increased in groups treated with statin and EPL compared with control while significantly decreased compared with NAFL group or NAFL and periodontitis group.

Table (3):Comparison between the studied groups according to OPG in the serum

| | Control (n= 7) | NAFL (n= 7) | NAFL and perio (n= 7) | Statin (n= 7) | EPL (n= 7) | p |
|-----------|-------------------|----------------|-----------------------------|---------------------------|--------------------------|-------------|
| OPG pg/ml | 54.2 ± 8.3 | 107.1 ± 16.3 | 135 ± 5.1 | 99.6 ± 12.3 ^{ac} | 95.6 ± 7.1 ^{ac} | <0.001 * |

p: p value for F test (ANOVA) for comparing between the different studied group

*: Statistically significant at $p \leq 0.05$, a: Significant with Control , b: Significant with NAFL c: Significant with NAFL+Perio

3.4 Histological examination



Fig. 1: Light micrograph (L.M.) of control group showing well-formed alveolar bone (alv) with relative smooth boundary facing the PDL, numerous osteocytes (arrows) and several resting lines are seen (R). Moderately distributed bone marrow spaces (bm) are observed



Fig..2: LM for intermediate rats with induced periodontitis showing signs of alveolar bone resorption with several reversal lines (rl). The PDL fibers are not well organized with areas of degeneration.

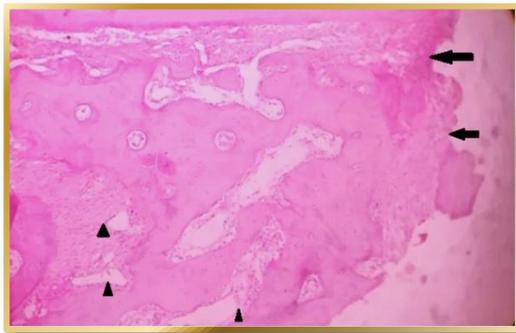


Fig..3: Periodontitis with fatty liver LM showing severe degeneration in PDL (arrow head) with severe bone destruction (arrow) * 100

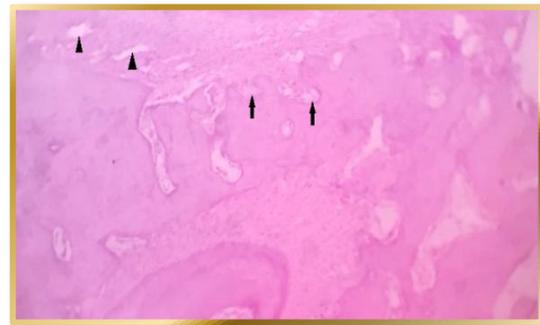


Fig. 4: LM Periodontitis with fatty liver receiving statin showing mild degeneration in the PDL fibers (arrow head), bone remodeling in the form of osteoclastic activity (arrow) H&e 100%

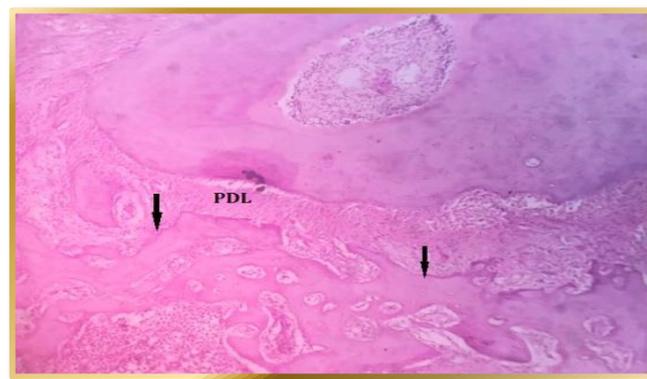


Fig. 5: Periodontitis with fatty liver receiving EPL. LM showing well-organized PDL fibers, bone trabeculae are well formed lined with well organized layer of osteoblast (arrow) HE 100

IV. Discussion

NAFLD is a chronic liver disease that occurs in people who drink little or no alcohol and is prevalent worldwide. Periodontal diseases may be implicated in the pathogenesis of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). Since periodontal pathogens, their endotoxins, and/or

cytokines released from the organisms could invade into the blood circulation and cause bacteremia, endotoxemia, and inflammation, such periodontal infections may also be implicated as an independent risk factor for NAFLD/NASH. For instance, the incidences of P. gingivitis infection have been found to be significantly higher in NAFLD patients as compared to healthy subjects [15,16], Therefore, early detection and treatment for NAFLD is important to prevent its progression to NASH, which may then develop into cirrhosis or liver cancer. So, it is important to elucidate the effect of essential phospholipid (EPL) for the treatment of NAFLD with periodontal disease and to compare it with statins. In the present study, we have utilized a well established animal model for NAFLD & periodontal disease and provided evidence that EPL treatment reduces lipids and alveolar bone loss in the animal model.

Our result showed that treatment with statin increase significantly liver enzymes (alanine, aspartate transaminases and γ GT) compared to NAFL group or group with NAFL and periodontitis "Table 1" these findings with agreement with (Argo et al 2008) [17] who found a rise of amino transaminases levels seen in the first six months of statins treatment for patient with NAFLD and he suggested that the aminotransferases elevation reflects the impact of statins therapy upon the hepatic metabolism, but unfortunately they are not a good marker for following the real impact upon the liver histopathology, also (Chalasanani et al., 2004) [18] found that the statins treatment may be accompanied by persistently elevated transaminases levels, more than three times the upper normal limit. With EPL treatment the values of ALT, AST and GGT fell to a greater extent than in the NAFLD group and NAFLD with periodontitis. Ohbayashi et al., 2004 and Ohbayashi et al., 2006 [19,20] confirmed with further 18 patients with NASH that long-term administration of EPL continues to improve hepatic function until completion of the study. First, significantly decreased transaminase levels were seen within 4-week treatment and continued over a 12-month therapy.

Statins are first-line therapy for the treatment of dyslipidaemia, a major risk factor for cardiovascular disease [21]. Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are drugs for lowering serum cholesterol, it interfere with cholesterol metabolism, a major pathway in the physiologic liver functions and, thus, might cause hepatotoxicity [22]. Our result revealed that treatment with statin significantly reduced total cholesterol, TG, LDL-c and improve HDL compared with NAFL group or NAFL and periodontitis group while treatment with EPL give a better result "Table 2". These results agreement with Li et al 2000 [23] who reported a positive influence of 1.8 g of EPL/day on total cholesterol (TC), triglycerides (TG), and transaminases, supported by significant improvement of the fatty liver (CT scan).

During the last years, EPL was compared to other compounds, discussed to be effective in NAFLD. The randomized, open controlled studies compared EPL to Gynostemma pentaphyllum gypenosides, to an extract from red yeast rice. EPL showed better relief of the clinical symptoms, improvement in serum TC and TG, ultrasound picture, and liver function [24]. Phosphatidylcholine (PC), a main component of EPL, is one of the most important support nutrients for the liver and is considered as a universal building block for cell membranes, which regulate the vast majority of the activities that make up life. PC helps recovery and maintenance of the consistency of the hepatocytes; it activates the phospholipid-dependent enzymes and improves lipid metabolism by accelerating synthesis of lipoproteins in the liver [25].

Osteoprotegerin (OPG) is a soluble protein, belonging to a super family of receptors of the tumor necrosis factor (TNF), acting as soluble pseudoreceptor for the receptor activator of ligand, nuclear factor κ β (RANKL, receptor-activator of nuclear factor κ β -ligand) to prevent activation of osteoclasts and thus bone resorption [26]. Our result showed significant elevation of OPG in both NAFL group and NAFL and periodontitis group compared to control group (table 3) these result with agreement with Fábrega et al 2005 who found a high level of OPG in 20 patients with alcoholic cirrhosis, compared with the control group population, also Moschen et al. (2005) [27] have found an increase in OPG levels in a sample of 87 cirrhotic patients, as compared with the control group. In 2010, a study published by Yilmaz and his collaborators indicated low levels of serum OPG in a sample of Turkish patients with NASH, as compared with those suffering from NAFLD and simple hepatic steatosis, but this study was conducted on a non-diabetic population (Yilmaz et al 2010) [28] OPG exerts osteoprotective effects by inhibiting osteoclast differentiation and activation and promoting osteoclast apoptosis [29]. Nevertheless, in relation to bone disease in the clinical setting, the association between OPG, bone density and fragility fractures remains controversial. In men, increased OPG levels have been associated with higher BMD of the lumbar spine [30]. Treatment with EPL or statin reduced the OPG levels compared to NAFL group or NAFL and periodontitis group, these results supported by histological findings "Figure 4 and 5" which show well-organized PDL fibers, well formed bone trabeculae lined with well organized layer of osteoblast.

V. Conclusion

EPL accelerates the improvement of biochemical parameters and pathological findings, such as liver function enzymes and lipid profile in rats with NAFLD and periodontitis. No relevant side effects were

reported. Additional adequately conducted randomized clinical trials on EPL versus statin or other active treatment are recommended to investigate the synergistic effects of EPL with other liver drugs.

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