Antioxidant and Anti-Inflammatory Efficacy of Optimized Ezetimibe Chitosan Nanoparticles against the Induced- Hyperlipidemia in Rats

Samya Mahmoud Ahmed¹, Amany Hegab ², Marwa H. Skukr³ and Soha Ismail³

¹Department of biochemistry, National Organization for Drug control and Research, Cairo, Egypt.  
²Department of development of pharmacology, National Organization for drug control and Research, Cairo, Egypt.  
³Department of Pharmaceutics, National Organization for drug control and Research, Cairo, Egypt.

Received: 20 Oct. 2021  Accepted: 20 Dec. 2021  Published: 30 Dec. 2021

ABSTRACT
Hyperlipidemia not only involves an elevation in serum lipids, but it is also an inflammatory disease, as excessive lipid accumulation is known to trigger local inflammatory responses with the production of oxidative stress. The developed and optimized formula of ezetimibe chitosan nanoparticles encompassing 30 mg EZE together with low molecular weight of chitosan at a ratio to TPP 1.75: 2, showed a considerable superior anti-hyperlipidemia when compared to the marketed product. This study investigated the role of optimized ezetimibe chitosan nanoparticles (optimized formula) in combating the oxidative stress and inflammation against induced - hyperlipidemia in rats. forty male rats were divided into two main groups: normal control (10 rats) and hyperlipidemia group where hyperlipidemia rat model was created by oral administration of cholesterol – cholic acid mixture in a ratio of 3:1 and feeding on 55gm butter /kg diet for ten weeks, then this group divided into three sub-groups; hyperlipidemia, ezetimibe treated group (0.9mg/kg b.wt) and optimized formula treated group (0.63mg/kg b.wt). Ezetimibe and optimized formula were prescribed for 4 weeks. Hyperlipidemia induced a significant increase in serum total cholesterol, triglycerides, aminotransferases (ALT and AST) in association with a significant decrease in serum total antioxidant capacity. Additionally, marked increase in malondialdehyde (MDA) level along with significant decline in reduced glutathione (GSH) content, antioxidant enzymes (glutathione S-transferase and Catalase) and nitric oxide (NO) production were observed in liver tissue of hyperlipidemia group. Hepatic tumor necrosis factor -alpha (TNF-α) was significantly increased in association with a significant decrease in interleukin 10 (IL-10). Treatment with Ezetimibe or the optimized formula effectively ameliorated these disorders and elicited their efficiency against oxidative stress and inflammation with the priority of the optimized formula. According to the obtained data, it can be concluded that treatment with optimized formula significantly relieved hyperlipidemia, by ameliorating the levels of aminotransferases with the suppression of oxidative stress and inflammation.

Keywords: Hyperlipidemia, Ezetimibe, Nanoparticle, Oxidative stress, Inflammation

1. Introduction
Hyperlipidemia, a life threatening metabolic disorder, is mainly manifested by an excess of fatty substances (cholesterol esters, cholesterol, phospholipids, triglycerides and/or cholesterol enriched lipoproteins) in the blood stream (Jang et al., 2012). Dietary cholesterol-induced hyperlipidemia leads to an inflammatory response and enhances oxidative stress in organs as the inflammation and oxidative stress are predominant under the condition of hyperlipidemia (Bondia-Pons et al., 2012). Excessive cholesterol intake provokes hepatic inflammation which plays a critical role in the progression of steatohepatitis, fibrosis, and finally cirrhosis and directly results in the progression of hepatitis (Harmon et al., 2011 and Ipsen et al., 2018). Inflammatory responses are promoted by the release of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) with
dysregulation of certain interleukins such as interleukin 10 (IL-10) (Ciebiada et al., 2013). Moreover, elevated oxidative stress with the increase of lipid peroxidation impairs the body's antioxidant status via the reduction of the endogenous antioxidant defense system, such as, reduced glutathione, catalase, and glutathione S-transferase (GST) (Marseglia et al., 2015; Fahmy et al., 2019; Tripathi et al., 2020 and Siddiqua et al., 2021). Therefore, it is preferred to use lipid-lowering drugs that control hyperlipidemia and subsequent oxidative stress and inflammation. Of these drugs, ezetimibe is prescribed because it is not only safe but also has a good tolerance and low adverse effect (Pikto-Pietkiewicz and Pasierb, 2006).

Ezetimibe (EZE) is a potent anti-hyperlipidemic agent that inhibits Niemann-Pick C1-Like1 (NPC1L1) protein - interposed cholesterol / phytosterol absorption in the apical membrane of the jejuna enterocyte (Jia et al., 2011). Interestingly, addition of EZE to statin can achieve the target value in the management of severe hypercholesterolemia, while using it lonely can treat mild hypercholesterolemia (Phan et al., 2012). Several studies have shown that EZE has pleiotropic effects (Kuroba et al., 2011 and Trocha et al., 2014). In addition to improving hyperlipidemia, EZE relieves oxidative stress in both human patient and mouse models (Sugizaki et al., 2014 and Hernandez-Mijares, 2016). Also, EZE exerts anti-inflammatory effects in mouse models (Lee et al., 2016).

Recently, antioxidants derived from natural polymers and saccharides have received much attention, with restrictions over the use of synthetic antioxidants. Chitosan, as a natural, safe, and cheap polysaccharide produced from chitin, possesses several favorable biological properties such as biodegradability, biocompatibility, and non-allergenicity (Wang et al. 2011 and Jafarizadeh-Malmiri et al., 2012). It has been reported that chitosan itself and many chitosan derivatives obtained by chemical modification have good antioxidant activity (Li et al., 2019; Li et al., 2020 and Affes et al., 2021). As is widely supposed, chitosan (CS)-based nanoparticles are very much acknowledged as a promising nanometric system that based on using biopolymers in biomedical applications as drug delivery because they are biocompatible, relatively non-toxic, biodegradable, and cationic in nature (Patel et al., 2012 and Prabaharan, 2015).

In our previous work (Shukr et al., 2019) we conducted a study aimed to develop and optimize ezetimibe chitosan nanoparticles by using $2^3\cdot 3^1$ factorial design, through the optimization of the effect of chitosan molecular weight, ratio of chitosan : tripolyphosphate (TPP) and drug concentration to achieve and improved the anti–hyperlipidemic activity of ezetimibe loaded CS/TPP nanoparticles. Our results revealed that, the optimized nanoparticles formula that composed of 30 mg EZE together with low molecular weight of chitosan at a ratio to TPP 1.75:2 displayed a significant increase of drug release with a more pronounced decreasing effect on the serum levels of TC, TG, Total lipids, LDL-C and VLDL-C indicating the superiority of this formula as anti-hyperlipidemia agent when compared to the marketed product (Shukr et al., 2019).

We proposed that ezetimibe loaded CS/TPP nanoparticles displayed its potential hyperlipidemia effect through the attenuation of inflammation and oxidative stress. Therefore, the aim of the present study is to investigate the beneficial role of this formula on hepatic oxidative stress and inflammation in hyperlipidemia in comparison with the effects of the marketed product of ezetimibe.

2. Materials and Methods

2.1. Drugs and Chemicals

EZE powder was kindly provided by Sabaa International Company (Egypt). Optimized EZE chitosan nanoparticles (optimized formula) was provided by Dr / Marwa H. Shukr and Dr / Soha Ismail, department of pharmaceutics, National Organization for drug control and research, Cairo, Egypt. EZE powder and optimized formula were received in a daily oral dose of 0.9 mg and 0.63 mg respectively /Kg b.w., calculated by extrapolation from the human dose (10 mg/day) (Ghosh, 2005). EZE powder was suspended in distilled water according to Jahangiri et al., (2016). Pure cholesterol and cholic acid were purchased from Sigma – Aldrich chemicals CO. (USA). In vitro diagnostic kits for determination of total cholesterol (TC), triglycerides (TG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Biodiagnostic (Cairo, Egypt). ELISA kits were provided by RayBiotech, (USA) for the determination of TNF-α and IL-10. Any other chemicals were of pure analytical grade.
2.2. Preparation of optimized formula

Preparation of optimized formula was carried out according to the method described in Shukr, et al., (2019).

2.3. Test animals and experimental design

A total of 40 male albino rats weighing 230 ± 20 gm, were obtained from the animal house of the National Organization for drug control and Research (NODCAR, Cairo, Egypt), and housed in stainless steel cages under good laboratory conditions (temperature 22± 3°C, 55% relative humidity and 12 h light/dark cycle) for two weeks as an acclimatization period with free access to water and fed on normal laboratory pellet diet. The present study was approved by the research ethics committee for experimental and clinical studies at NODCAR (NODCAR /1/2/2020)

Rats were randomly divided into two main groups: Group (1): fed on normal diet and served as normal control group (10 rats), the other rats were received a mixture of cholesterol-cholic acid and saturated fats for 10 weeks to obtain a hyperlipidemic model. Thereafter, hyperlipidemic rats were divided into: Group (11): hyperlipidemic control group .Group (111): received a daily oral dose of ezetimibe "0.9 mg /kg b.w." for 4 weeks. Group (1V): received a daily oral dose of optimized formula "0.63 mg /kg b.w." for 4 weeks.

2.3.1 Induction of hyperlipidemia

10%/ Kg b.w. from a mixture of cholesterol-cholic acid at a ratio of 3:1 were used orally as an inciting operator for hyperlipidemia that was enhanced by supplementation with 55 gm butter /Kg pellet diet (Hussein et al., 2014). After ten weeks, hyperlipidemia was affirmed by increasing serum total cholesterol and triglycerides levels as the most practical means of revealing hyperlipidemia. Treatment with EZE or optimized formula was started after incidence of hyperlipidemia for four weeks.

2.3.2 Blood and tissue samples

At the end of 4 weeks of treatment, after overnight fasting, blood samples were obtained from retinobulbar venous plexus and serum was separated by centrifugation at 3000rpm for 15 minutes and stored at -20 for further biochemical investigation. Thereafter, animals will be euthanized by decapitation and the liver was excised, washed with cold saline and stored at -80 °C for further biochemical analysis.

3.1 Biochemical analysis

The serum TC, TG, ALT and AST were assessed by using in vitro diagnostic kits (Biodiagnostic, Cairo, Egypt) following manufacturer's instructions. Serum total antioxidant capacity (TAC) was determined according to the method of Benzie and Strain, (1996).

3.1.1 Determination antioxidants markers in liver homogenate

MDA level as a marker for lipid peroxidation was assessed according to the method of Uchiyama and Miwara, (1978), GSH content was determined according to the method of Beutler et al., (1963), GST activity was determined according to the method of Habig et al., (1974), CAT activity was determined according to the method of Aebi , (1984), NO level was determined according to the method of Miranda et al., (2001) and total protein according to the method of Lowery et al., (1951).

3.1.2 Determination of pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines

The levels of TNF-α and IL-10 were assayed by an in vitro enzyme – linked immunosorbent assay (ELISA) kits (RayBiotech, USA) in liver homogenate according to manufacturer's instructions.

Statistical analysis

The data were statistically analyzed using statistical package for social science (SPSS version 19 software). Data were expressed as mean ± SE. The statistical evaluation of the difference between the groups mean values was assessed by one-way analysis of variance (ANOVA) followed by Duncan's test and values of p<0.05 were considered as statistically significant.
Results

At the end of the experimental period, TC and TG levels significantly (p<0.05) increased in hyperlipidemic control group by 155% and 226 % respectively as compared to the normal control group. Also, ALT and AST activities significantly increased in hyperlipidemic control group by 56.1 % and 30.44 % respectively as compared to the normal control group. Meanwhile, treatment with EZE or optimized formula showed a significant (p <0.05) decrease in serum TC, TG, ALT and AST levels by (47.29% , 52.88%), (51.4%, 59.7%), (15.99%, 12.37%) and (22.33% , 27.69%) respectively as compared to the hyperlipidemic control group (Figs .1A and 1B).

The results presented in Tables (1) and (2) revealed that, the administration of cholesterol–cholic acid mixture and saturated fat diet throughout the experimental period induced oxidative stress in rat's liver that is indicated by the significant (p <0.05) increase in MDA level (more than threefold the level of normal control group) in association with a significant (p <0.05) decrease in the levels of TAC, GSH, NO, CAT and GST as compared to normal control group. On the contrary, treatment with EZE or optimized formula inhibited the MDA formation by (31.25% , 23.88%) respectively and significantly elevated the levels of CAT by ( 25.28 % , 57.93%) , GST by (112.22%, 105.9%) ,TAC by ( 67.97% , 76.61%) , GSH by (48.74 % , 43.06%) and NO by ( 67.59%, 69.52%) respectively as compared to the hyperlipidemia group .

Table 1: Effect of four weeks treatment with ezetimibe powder and optimized formula on MDA, TAC, NO and GSH levels in the induced- hyperlipidemic rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nM/g tissue)</th>
<th>TAC (μM/L)</th>
<th>NO (μM/g tissue)</th>
<th>GSH (μM/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>554.12±39.76</td>
<td>757.92±11.04</td>
<td>124.13±1.88</td>
<td>13.94±0.22</td>
</tr>
<tr>
<td>Group II</td>
<td>1755.21±72.08</td>
<td>407.22±14.78</td>
<td>93.50±7.11</td>
<td>9.15±0.15</td>
</tr>
<tr>
<td>Group III</td>
<td>1206.78±41.89</td>
<td>683.99±10.54</td>
<td>156.70±3.26</td>
<td>13.61±0.24</td>
</tr>
<tr>
<td>Group IV</td>
<td>1336.01±59.36</td>
<td>719.20±12.03</td>
<td>158.50±6.59</td>
<td>13.09±0.42</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE (n=6). ANOVA test followed by Duncan's multiple comparisons between groups at P <0.05 were employed. The presence of different letters means significant difference between groups in the same column.

Our results also showed that, feeding hyperlipidemic regimen caused a well-marked elevation in the level of hepatic TNF-α along with a significant (p<0.05) decline in the level of IL-10 as compared to normal control group. On the other hand, treatment with EZE or optimized formula causes significant ((p<0.05) decrease in TNF-α and significant increase in IL-10 level as compared to the hyperlipidemia group (Table 2).
It may be pointed out that, the obtained results not only show that optimized formula (with low dose than EZE) exhibit nearly the same effect as EZE but it exceed it as observed in the significant increase in CAT activity and IL-10 level and a significant (p<0.05) decrease in the level of TNF-α (Table 2).

<table>
<thead>
<tr>
<th>Groups</th>
<th>CAT (U/mg protein)</th>
<th>GST (U/mg protein)</th>
<th>TNF-α (pg / mg tissue)</th>
<th>IL-10 (pg / mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>19.52±1.23</td>
<td>507.71±16.20</td>
<td>33.38±2.28</td>
<td>146.11±8.89</td>
</tr>
<tr>
<td>Group II</td>
<td>14.95±1.08</td>
<td>371.42±9.84</td>
<td>142.07±6.38</td>
<td>46.12±2.54</td>
</tr>
<tr>
<td>Group III</td>
<td>18.73±0.96</td>
<td>788.25±12.98</td>
<td>91.03±7.58</td>
<td>62.35±3.29</td>
</tr>
<tr>
<td>Group IV</td>
<td>23.61±1.15</td>
<td>764.76±27.66</td>
<td>65.11±4.03</td>
<td>77.68±4.58</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE (n=6). ANOVA test followed by Duncan's multiple comparisons between groups at p<0.05 were employed. The presence of different letters means significant difference between groups in the same column.

4. Discussion

Cardiovascular diseases are as yet the main source of morbidity and mortality worldwide. Hyperlipidemia as a complex lipid metabolism disorder was reported to be one of the major common and important risk factors in the development and progression of cardiovascular diseases such as atherosclerosis and hypertension (Navar - Boggan et al., 2015). In the current study as well as in our previous study (Shukr, et al., 2019), oral administration of cholesterol-cholic acid mixture and feeding on saturated fat diet for ten weeks was used to create the hyperlipidemia rat model and elevation of serum TC and TG levels indicated the successful induction of hyperlipidemia in rats. Our results are consistent with several studies reported the creation of similar hyperlipidemia model (Lassoued et al., 2018; Ontawong et al., 2019; Wu et al., 2020 and Du et al., 2021). In our previous work, the developed and optimized ezetimibe chitosan nanoparticles exhibited a more potent anti-hyperlipidemia efficacy than that displayed by ezetimibe marketed product, through lowering the serum levels of TC, TG, Total lipids, LDL-C and VLDL-C (Shukr, et al., 2019). The hypolipidemic effect of EZE may be attributed to the inhibition of intestinal absorption of biliary and dietary cholesterol by suppressing Niemann-pickC1-like1 protein which is a cholesterol transporter residing in small intestine (Jia et al., 2011).

As it well known that liver plays a central role in lipid metabolism therefore, in the present study we investigated the liver function marker ALT and AST with the investigation of liver redox state under the influence of high cholesterol and triglyceride levels. In the present study, the increased aminotransferases level in the serum of hyperlipidemic rats indicates alterations in membrane integrity and / or permeability (Welch-White et al., 2013). Excessive accumulation of lipids in hepatocytes due to an imbalance between lipid formation and lipid degradation, caused the increased levels of aminotransferases result from leakage from damaged hepatic cells and can be used as markers of liver injury (Yadav et al., 2009). Meanwhile, treatment with EZE or optimized formula decreased the level of these enzymes which may be related to the hypolipidemic activity of them and suggested that they have liver protective effect (Qian et al., 2016; Dizaye and Mohammed, 2019).

In the present study, administration of hyperlipidemic diet enhanced the mechanism involved in tissue damage (Ponce-Canchihuaman et al., 2010 and Ma et al., 2012); in particular the oxidative stress, that evidenced through the increased concentration of hepatic MDA in association with decreased of GSH content as well as the antioxidant enzyme activities namely, Catalase and GST.

Oxidative stress regarded as an early event in the development of hyperlipidemia and it has been proposed to be the mechanism through which hyperlipidemia stimulates tissue damage (Ponce-Canchihuaman et al., 2010 and Ma et al., 2012). ROS could oxidized LDL-C to form Ox-LDL-C that is associated with the incidence of hyperlipidemia (Wu et al., 2013). In the current work the biochemical changes caused by hyperlipidemic diets were confirmed by the obtained results, that revealed a significant increase in MDA level, along with a significant decrease in the levels of total antioxidant capacity (Li et al., 2013) and antioxidant system (reduced glutathione, CAT, GST). The ability of this defense system is obviously declined in hyperlipidemia, resulting in incapable
scavenging of free radicals, that lead to tissue damage (Prasanna and Purmima, 2011). This finding may be attributed to the presence of accumulated lipid peroxides and reflected the possible increase demand in GSH utilization for neutralizing free radicals under oxidative stress. Also, the obtained depletion in the endogenous antioxidant enzymes as CAT and GST under the influence of hyperlipidemia may be attributed to the inactivation of these enzymes with decreasing their ability for free radical scavenging that leading to development of oxidative stress (Smathers et al., 2011). Our findings are consistent with the results of Naik et al. (2018) and Zeng et al. (2019) that demonstrated the enhancement of oxidative stress after hyperlipidemic diet exposure. Herein, treatment of hyperlipidemic rats with EZE or optimized formula resulted in an improvement in MDA, TAC and antioxidant system. These results indicated that EZE or optimized formula ameliorate oxidative damage caused by hyperlipidemia and confirmed the antioxidative effect of EZE and the optimized formulation. These findings may be attributed to the hypolipidemic effect of EZE that decreased cholesterol influx to the cells thereby reducing cholesterol-induced oxidative stress. Also, EZE could attenuate oxidative stress by down regulation of NADPH oxidases, cytochrome P4502E1 and beta oxidation (Sugizaki et al., 2014). Our results are in consistent with the studies of Trocha et al., (2014) and Dizaye and Mohammed (2019).

Accumulation of reactive oxygen species not only associated with lipid peroxidation but also enhanced endothelial dysfunction, in terms of reducing endothelium-derived NO, leading to vasoconstriction (Forsterrman et al., 2017). The current study shows that administration of hyperlipidemic diet decrease NO production in hyperlipidemic group as compared to normal group that is consistent with the study of Liu et al., (2014). Hyperlipidemia is associated with the production of oxidized LDL (ox-LDL) that induced endothelium damage leading to decreasing in endothelial NO synthase (eNOS) and subsequent decrease in the production of NO (Deepa and Varalakshmi, 2005).

In the current study, treatment with EZE or optimized formula caused an increase in NO production, which could be attributed to the decreasing of asymmetric dimethylarginine (ADMA) level that is an endogenous inhibitor of endothelial nitric oxide synthase (eNOS). Thus, EZE inhibit endothelial dysfunction by enhancing NO production through the increase of eNOS and this support their protective role against vascular injury as explained by Fukuda et al. (2010).

In the present study, the obtained increase in the pro-inflammatory cytokines TNF-α that was associated with significant decrease in the anti-inflammatory cytokines IL-10 proved the induction of inflammatory response under the influence of hyperlipidemia. These findings are in compatible with the results conducted on the same subject (Awad et al., 2016 and Yuan et al., 2019). TNF-α (classic cytokine that enhanced in response to systemic inflammation and affected on lipid metabolism so it is implicated in the development of hyperlipidemia and atherogenesis), and IL-10 (an immunomodulatory cytokines that produced by Th2 cells and able to inhibit the inflammatory response) are able to affecting each other and they demonstrate a reversible relationship (Chen et al., 2009 and Huang, 2010). Growing evidences correlate between consumption of high cholesterol diets and chronic inflammatory state. This presumption plays an important and modulating role in the pathogenesis of various diseases (Ciebiada et al., 2013). Reactive oxygen species produced due to accumulation of lipid, activate nuclear factor-kappa (NF-kB) that increase the expression of TNF-α in the liver (Park et al., 2010) and these cytokines can stimulate further production of ROS and hence inducing liver damage (Braunersreuther et al., 2012). Also, induction the expression of adhesive molecules by ox-LDL leads to the secretion of cytokines (Rocha and Libby, 2009). Subsequently, the regulation of these cytokines could inhibit the progression of atherosclerosis. Indeed, in our study, treatment with EZE or optimized formula decreased TNF-α and increased IL-10. The mechanism implied the effect of EZE or optimized formula on IL-10 may be related to the positive influence of EZE on the inflammatory process as reported by Lee et al., (2016). Similar results were obtained in the studies of Chan et al. (2010) and Tie et al., (2015), in which EZE significantly decreased TNF-α level supporting the hypothesis that EZE has anti-inflammatory and antiatherogenic activity. The protective effect of EZE against inflammation might be due to the inhibition of cholesterol inflow, including oxidized cholesterol into the liver from the intestine and / or by the direct effect on the liver (Deushi et al., 2007) or through decreasing oxidative stress (Shusuke et al., 2010). Also, decreasing in pro-inflammatory markers may reduce the progression of atherosclerosis and release of the cytokines by inflammatory accumulated cells that causes an improvement in endothelial function and attenuates endothelial activation.
Obviously, the nano-aided delivery system of poorly soluble drugs can improve the solubility, oral bioavailability, biodiffusion, targeted drug delivery and decreased dose of the drug (Muller and Akkar, 2004; Chan, 2006 and Zhang et al., 2008). Our results demonstrated that treatment with the developed optimized nanoformula exhibits a hepatoprotective effect by ameliorating oxidative stress and decreasing the release of inflammatory cytokines.

4. Conclusion

Data of the current study proved that, the optimized formula displayed a well-marked antioxidant and anti-inflammatory potency. Apart from the significant effect of optimized formula on CAT, TNF-α and IL-10 as compared to EZE, the data also revealed that oral administration of optimized formula (0.63mg / kg b.wt) or ezetimibe (0.9 mg/kg b.wt) showed to some extent the same hepatoprotective effect against lipid peroxidation and inflammatory markers in hyperlipidemic rats. Therefore, the use of optimized formula could be beneficial for the prevention of hyperlipidemia.

References


