

Ameliorative Potential of Thiazolidinediones and Statins on Experimentally Induced Non-Alcoholic Steatohepatitis

Ahmed M Kabel^{1,2,*}, Hany M Borg³

¹Department of Clinical Pharmacy, College of Pharmacy, Taif University, Taif, Saudi Arabia

²Department of Pharmacology, Faculty of Medicine, Tanta University, Tanta, Egypt

³Department of Physiology, Faculty of Medicine, Kafrelsheikh University, Egypt

*Corresponding author: drakabel@gmail.com

Received October 20, 2014; Revised December 01, 2014; Accepted December 05, 2014

Abstract Non-alcoholic steatohepatitis (NASH) is a chronic liver disease that may progress to advanced fibrosis and cirrhosis. Yet, its pathogenesis is not fully understood. The aim of this work was to study the possible protective effect of atorvastatin and pioglitazone on non-alcoholic steatohepatitis induced by high fat diet (HFD) in rats. Sixty male albino rats were divided into two main groups, the normal control group (ten rats) and HFD group (fifty rats) which was further subdivided into five subgroups (ten rats each) one of them received HFD+normal saline (NASH group), the 2nd received HFD + vehicle (gum tragacanth), the 3rd received HFD+atorvastatin, the 4th received HFD+ pioglitazone and the last group received HFD+atorvastatin and pioglitazone. At the end of the study, animals were killed and blood samples were collected for estimations of serum ALT, AST, ALP, TNF- α , triglycerides, total cholesterol, glucose, insulin and HOMA-IR. Also, samples of liver were taken and studied for hepatic triglycerides, malondialdehyde, free fatty acids and histopathological analysis. Atorvastatin lowered efficiently serum ALT, AST, ALP, TNF- α , total cholesterol, triglycerides, hepatic triglycerides, malondialdehyde and free fatty acids, together with marked improvement in histopathology of liver steatohepatitis, but, produced insignificant effects on fasting blood glucose, serum insulin and HOMA-IR. On the other hand, administration of pioglitazone, whether alone or in combination with atorvastatin induced significant improvement of all the above mentioned parameters. In conclusion, if the prominent feature in NASH is insulin resistance, we recommend the use of pioglitazone, while atorvastatin will be needed if the prominent features is hyperlipidemia and both drugs simultaneously if there are both hyperlipidemia and insulin resistance.

Keywords: statins, thiazolidinediones, non-alcoholic steatohepatitis, rats

Cite This Article: Ahmed M Kabel, and Hany M Borg, "Ameliorative Potential of Thiazolidinediones and Statins on Experimentally Induced Non-Alcoholic Steatohepatitis." *World Journal of Nutrition and Health*, vol. 2, no. 4 (2014): 52-57. doi: 10.12691/jnh-2-4-2.

1. Introduction

Non-alcoholic steatohepatitis (NASH) is a chronic disorder that is increasingly recognized as being very common. NASH has a potential to progress to advanced fibrosis and cirrhosis. Oxidative stress, excessive hepatocyte apoptosis and toxins are implicated as the main factors in the pathogenesis of progressive NASH (Metha et al., 2002; Imeryuz et al., 2007; Tahan et al., 2007; Takaki et al., 2014).

The pathogenesis of NASH is not fully understood, and the "two hit" hypothesis proposed by Day and James (1998) remains the prevailing theory. The "first hit" is the development of steatosis because of prolonged over nutrition that leads to accumulation of liver free fatty acids and triglycerides. In the "second hit", steatosis progresses to NASH and this is associated with factors such as

oxidative stress, mitochondrial dysfunction and inflammatory cytokines.

To date, there is no consensus on effective therapy (Torres & Harrison, 2008). Promising treatments for NASH include antioxidants, hepatoprotective agents, antidiabetic agents and angiotensin II receptor antagonists (Yokohama et al., 2004; Comar & sterling, 2006).

Atorvastatin, an inhibitor of 3-hydroxy -3-methyl glutaryl CoA reductase enzyme, has been reported to be effective in patients with NASH and dyslipidemia (Gomez-Dominguez et al., 2006; Kabel et al., 2013). It was demonstrated that therapy with atorvastatin in NASH patients with dyslipidemia was effective for reduction of serum aminotransferases and lipid contents. However, the efficacy of atorvastatin treatment for the histological changes was not available.

Pioglitazone, one member of thiazolidinediones, is used to improve insulin resistance in type II diabetes mellitus. Pioglitazone is a ligand of the peroxisomal proliferator activated receptor γ (PPAR- γ) which has been reported to

improve hepatic steatosis, alanine aminotransferase elevation and insulin sensitivity in patients with NASH (Kawaguchi et al., 2004; Kallwitz et al., 2008).

The aim of the present work was to investigate the possible protective effects of atorvastatin and pioglitazone on nonalcoholic steatohepatitis induced by high fat diet in rats.

2. Materials and Methods

2.1. Drugs and Chemicals

- Atorvastatin tablets 40 mg (Lipitor, a product of Pfizer).
- Pioglitazone tablets 15 mg (Glustin, a product of Lilly, USA).
- Gum tragacanth powder (Sigma Chemical Co. USA).
- Commercially available kits, Diamond diagnostics for estimation of ALT, AST, ALP, triglycerides, total cholesterol and blood glucose.
- Commercially sandwich Elisa kits for rats according to manufactures instructions (Biosource, International Camarillo California, USA) for determination of TNF α .
- Insulin enzyme linked immunosorbant assay (ELISA) using kits for determination of insulin (DRG diagnostic, Germany).

2.2. Animals and Procedures

Sixty male albino rats each weighing 100-120 grams were used through this study, they were kept under similar housing conditions and were subdivided into two main groups as follows:

Group 1: Consisted of ten rats, were fed standard diet and received saline at a dose of 1ml/ rat by gastric tube daily throughout the study and served as normal control group.

Group 2: Consisted of 50 rats and were fed a high fat diet (HFD) containing 80.5% basal feedstuff, 2% cholesterol, 7% lard, 10% yolk flour and 0.5% bile salt for 8 weeks (Xu et al., 2006). They were further subdivided into five subgroups as follow:

Group 2a: rats were fed HFD and normal saline by gastric tube daily and served as NASH group.

Group 2b: rats were fed HFD with 1% tragacanth mucilage by gastric tube daily.

Group 2c: rats were fed HFD with atorvastatin-tragacanth suspension, at daily dose of 8mg/kg by gastric tube for 8 weeks (Kamada et al., 2003).

Group 2d: rats were fed HFD with pioglitazone-tragacanth suspension, at daily dose of 4 mg/kg by gastric tube for 8 weeks (Xu et al., 2006).

Group 2e: rats were fed HFD with atorvastatin and pioglitazone by gastric tube daily with the same previous dose for 8 weeks.

At the end of the experiment, animals were killed by decapitation after 12 hours of fasting and rats were subjected to the following procedures:

3. Biochemical Procedures

Blood was collected and centrifuged for estimation of the following:

- Serum alanine and aspartate transaminases (ALT, AST) (Reitman & Frankel, 1957).
- Serum alkaline phosphatase (ALP) (Reitman & Frankel, 1957).
- Serum triglycerides (Fossati & Prencipe, 1980).
- Serum total cholesterol (Richmoud, 1973).
- Fasting blood glucose level (Trinder, 1969).
- Serum TNF- α (Stepaniake et al, 1995 ; Song et al., 1998).
- Fasting serum insulin level (Ishikawa et al., 1989).
- Homeostasis model assessment-insulin resistance was calculated (HOMA-IR) = [fasting glucose (mmol/L) X fasting insulin (μ U/ml)] /22.5 (Bonora et al., 2000).

4. Tissue Homogenization

Liver samples were homogenized for measurement of malondialdehyde (MDA) (Ohkawa et al., 1979), triglycerides (Blighard & Dyer, 1995) and free fatty acids (Itaya & Kadowaki, 1969). Protein content of the liver tissue was determined by the method of Lowry et al., (1951).

5. Histopathology

Sections 5- μ m thick of the right lobe of all rat liver, fixed in 10% formalin for 24 hours and embedded in paraffin, were processed for haematoxylin and eosin stain for histopathological studies.

- Mild steatosis (+) less than 25% hepatocytes in sections in 10 high power field showed fatty changes.
- Moderate steatosis (++) more than 50% hepatocytes in sections in 10 high power field showed fatty changes.
- Sever steatosis (+++) nearly all hepatocytes showed massive fatty changes in sections in 10 high power field.
- Hepatic inflammation was quantified as the ratio of the area of inflammatory foci (defined as groups of five or more inflammatory cells) to the total area of the section (Leclercq et al., 2004).

5.1. Statistical Analysis

Data were presented as mean \pm SEM, data were analyzed by one way analysis of variance (ANOVA) using student t-test, differences between the means of different groups were considered significant at a level of $p < 0.05$.

6. Results

6.1. Biochemical Findings

a. Feeding rats with HFD for 8 weeks (NASH), induced significant ($P < 0.05$) increase in serum ALT, AST, ALP, total cholesterol, triglycerides, TNF- α , blood glucose, serum insulin and HOMA-IR when compared to normal control group (Figure 1, Figure 2).

b. Administration of atorvastatin with HFD for 8 weeks produced significant ($P < 0.05$) decrease in serum ALT, AST, ALP, total cholesterol, triglycerides and TNF- α and non significant effect ($P > 0.05$) on fasting blood glucose, serum insulin or HOMA-IR, when compared to NASH group (Figure 1, Figure 2).

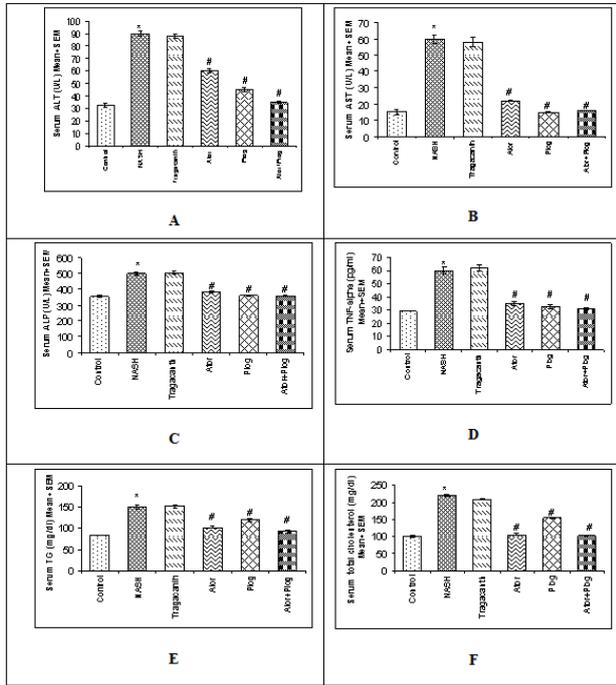


Figure 1. A (Serum ALT); B (Serum AST); C (Serum ALP); D (Serum TNF-alpha); E (Serum triglycerides) and F (Serum total cholesterol) in the studied groups

* Significant compared to the control group ($P < 0.05$).

Significant compared to NASH group ($P < 0.05$).

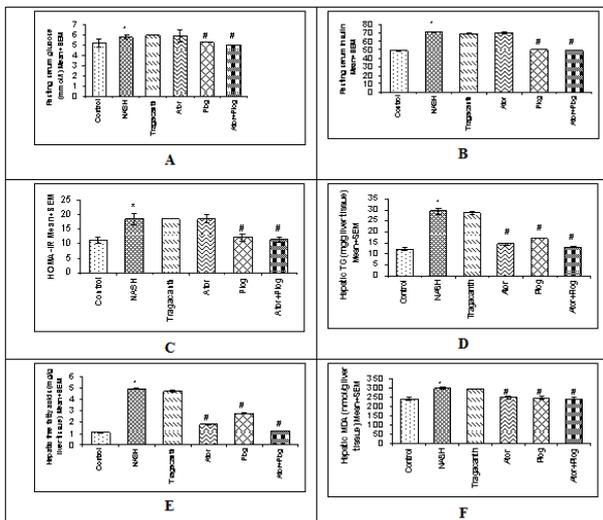


Figure 2. A (Fasting serum glucose); B (Fasting serum insulin); C (HOMA-IR); D (Hepatic triglycerides); E (Hepatic free fatty acids) and F (Hepatic malondialdehyde) in the studied groups

* Significant compared to the control group ($P < 0.05$).

Significant compared to NASH group ($P < 0.05$).

c. Administration of pioglitazone with HFD or its combination with atorvastatin with HFD for 8 weeks produced significant ($P < 0.05$) decrease in serum ALT, AST, ALP, total cholesterol, triglycerides, TNF- α , blood

glucose, serum insulin and HOMA-IR when compared to NASH group (Figure 1, Figure 2).

d. Administration of tragacanth mucilage with HFD for 8 weeks produced insignificant effect ($P > 0.05$) on the above mentioned parameters when compared to NASH group (Figure 1, Figure 2).

6.2. Tissue Homogenization Findings

a. Feeding rats with HFD for 8 weeks produced significant increase in Hepatic MDA, hepatic triglycerides and hepatic free fatty acids when compared to the normal control group (Figure 2).

b. Administration of atorvastatin or pioglitazone alone, or in combination, with HFD for 8 weeks, produced significant ($P < 0.05$) decrease in hepatic MDA, hepatic triglycerides and hepatic free fatty acids when compared to NASH group (Figure 2).

c. Administration of tragacanth mucilage with HFD for 8 weeks produced non significant ($P > 0.05$) effects when compared to NASH group (Figure 2).

6.3. Histopathological Findings

a. Feeding rats with HFD for 8 weeks (NASH group) showed diffuse steatosis together with marked inflammatory infiltration (Figure 4).

b. Administration of atorvastatin or pioglitazone with HFD for 8 weeks showed minimal steatosis with minimal inflammatory infiltration (Figure 5, Figure 6).

c. Administration of atorvastatin and pioglitazone in combination with HFD for 8 weeks showed more or less normal liver tissues with normal lobular architecture. (Figure 7).

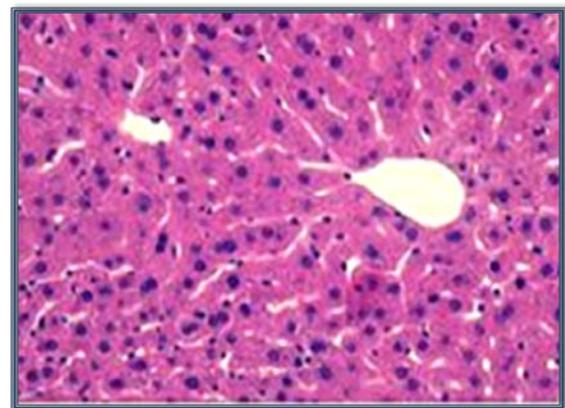


Figure 3. Section from liver of normal group showed normal liver cells with normal lobular architecture (H&E X 250)

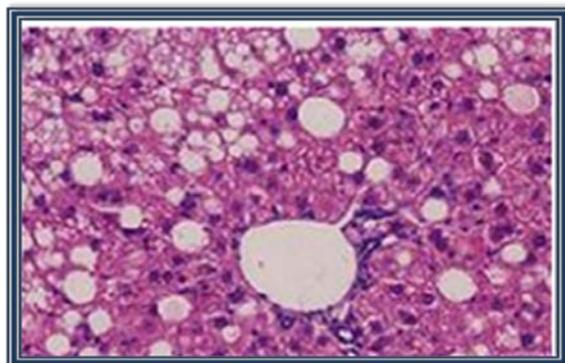


Figure 4. Section from liver of NASH group showed extensive steatosis with marked inflammation (H&E X 250)

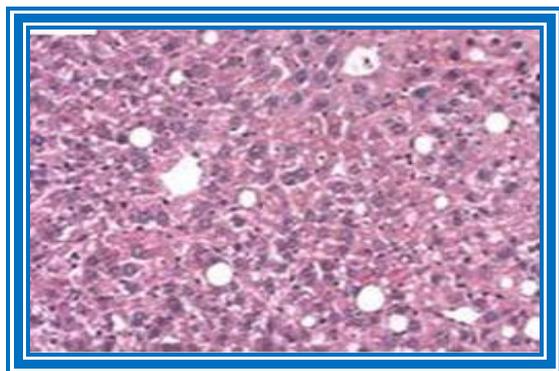


Figure 5. Section from liver of atorvastatin treated group showed minimal steatosis with no inflammation (H&E X 250)

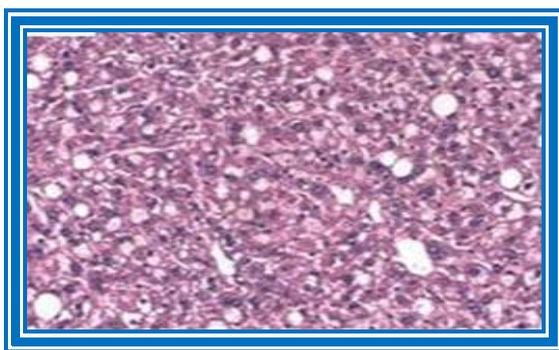


Figure 6. Section from liver of pioglitazone treated group showed minimal steatosis with no inflammation (H&E X 250)

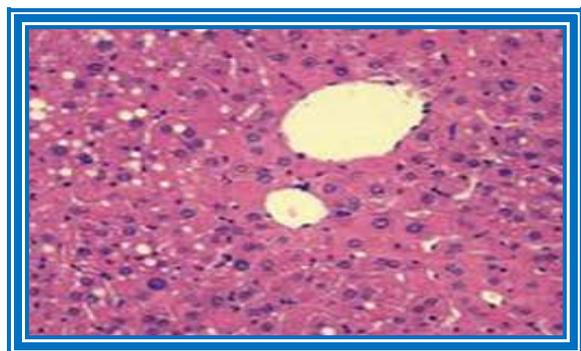


Figure 7. Section from liver of atorvastatin + pioglitazone treated group showed minimal steatosis with no inflammation and more or less normal lobular architecture (H&E X 250)

7. Discussion

Non-alcoholic steatohepatitis (NASH) is common and may progress to cirrhosis and its complications. The pathogenesis of steatosis and cellular injury is thought to be related mostly to insulin resistance and oxidative stress (Adams & Angulo, 2006). Obesity and diabetes are frequently associated with non-alcoholic steatohepatitis (Lieber et al., 2004).

The high fat diet model induced NASH that was used in this study correlates with most of pathogenic findings known to be responsible for the occurrence of hepatic steatosis associated with obesity and insulin resistance in humans (Xu et al., 2006). Also, administration of high fat diet for 8 weeks produced marked elevation of liver transaminases and ALP when compared to the normal control group. These results are in accordance with Angulo et al., (1999) and Fan et al., (2003). They stated that mild to moderate elevation in serum ALT, AST and

ALP are the most common laboratory abnormalities found in patients with NASH.

In the present study, HFD produced significant increase in serum insulin and fasting blood glucose levels together with marked increase in insulin resistance as reflected by increase in HOMA-IR. This is in agreement with Lieber et al., (2004) and Ping et al., (2006). They stated that rats fed with high fat diet had significantly higher plasma insulin concentrations and HOMA-IR which reflected insulin resistance in animal model of NASH.

The molecular pathogenesis of insulin resistance in NASH seems to be multifactorial and several molecular targets involved in the inhibition of insulin action have been identified. These include PC1 (a membrane glycoprotein) that has a role in insulin resistance through reduction of insulin stimulated tyrosine kinase activity (Maddux et al., 1995), Leptin which induces dephosphorylation of insulin receptor substrate1 (Cohen et al., 1996), fatty acids that inhibit insulin stimulated peripheral glucose uptake (Boden, 1997) and TNF- α which down-regulates insulin induced phosphorylation of insulin receptor substrate 1 (Hotamisligil et al., 1996).

In the present work, HFD produced significant increase in hepatic MDA and TNF- α . This is in agreement with Sanyal et al., (2001); Koruk et al., (2004); Lieber et al., (2004); Seki et al., (2005) and Tahan et al., (2007) who demonstrated the role of oxidative stress, increased cytokine activity and mitochondrial dysfunction in the pathogenesis of NASH. Moreover, Paradise et al., (2001) explained these results according to the "second hit hypothesis" where the first hit is steatosis that increases the sensitivity of the liver to secondary insult (Day & James, 1998). Cytokines, mitochondrial dysfunction, oxidative stress and lipid peroxidation are the main "second hits" in the induction of primary NASH. Oxidative stress in particular with subsequent lipid peroxidation and generation of reactive oxygen species are to be prominent in NASH with subsequent generation of proinflammatory cytokines such as TNF- α . On the other hand, insulin resistance is also associated with hypertrophy of the microsomal oxidants function due to increased activity of cytochrome P-450 system. In turn, this is caused by loss of the insulin inhibitory effect and to up regulation mechanisms (Robertson et al., 2001). This condition may lead to intracellular oxidative stress if there is an imbalance between pro-oxidant and antioxidant molecules (Chitturi & Farrell, 2001).

In the present study, HFD produced significant increase in hepatic triglycerides and free fatty acids. These results are in accordance with Gianluca et al., (2006). The most accepted explanation is offered by Browning & Horton (2004) who explained the marked increase in hepatic triglycerides and free fatty acids on the basis of visceral obesity and insulin resistance, mostly mediated by adipokines such as TNF- α that result in increase FFA release from adipocytes with consequent enhancement of lipid delivery to the liver. Moreover, Bugianesi et al., (2004) postulated that the genetic defect is the primary factor responsible for insulin resistance and visceral obesity. Progression from fatty liver to steatohepatitis may be due to imbalance between proinflammatory and anti-inflammatory cytokines, triggering the formation of reactive oxygen species and intrahepatic lipid peroxidation (Tamura & Kawamori, 2006).

In this study, administration of atorvastatin with HFD was accompanied with insignificant changes in serum glucose or insulin levels while produced significant decrease in liver enzymes, TNF- α , hepatic triglycerides, hepatic malondialdehyde levels with marked improvement in hepatic steatosis. This is in agreement with Hyogo et al., (2008) who stated that atorvastatin was highly effective in lowering elevated liver transaminases together with marked reducing effect on hepatic lipids and TNF- α when given to patients suffering from NASH with hyperlipidemia. This may be explained by the ability of atorvastatin to induce peroxisome proliferator-activated receptor (PPAR- α and PPAR- γ) (Grip et al., 2002). The PPAR-activation increases β oxidation of fatty acids followed by decrease of fatty acids available for triglycerides synthesis, and thus decrease the synthesis of triglycerides in the liver. The PPAR- γ has been demonstrated to attenuate the inflammatory response by inhibiting the production of TNF- α in monocytes (Grip et al., 2002).

Also, administration of pioglitazone with HFD in this study was accompanied with significant decrease in serum liver enzymes, TNF- α , insulin resistance, hepatic triglycerides, free fatty acids and hepatic malondialdehyde. This is in accordance with Tahan et al., (2007); Da Silva Morais et al., (2009) who reported that PPAR- γ have marked insulin sensitizing effects and anti-inflammatory effects manifested by significant decrease in inflammatory markers as TNF- α . The exact mechanism that explains how pioglitazone improve steatohepatitis is still unknown. The two-hit theory has been proposed to explain the mechanism for NASH (Da Silva Morais et al., 2009) and pioglitazone has been suggested to improve NASH by inhibiting the first hit. Many authors proposed that thiazolidinediones (TZD) stimulate adipocyte proliferation and fat mass expansion favoring the storage of fat in adipose tissue, sparing peripheral tissues, such as liver from fat accumulation and lipotoxicity (Teranishi et al., 2007). Other researchers suggested that TZD may modulate adipocytokine release, in particular, they induce the production of adiponectin, favoring increased fatty acid oxidation, decreased lipogenesis and decreased inflammation (Miyazaki et al., 2004). Also, TZD may modulate lipid metabolism, hepatic inflammation and fibrosis by activation of peroxisome proliferator-activated receptor gamma (PPAR- γ), PPAR- α , adiponectin or other cytokines (Von Knethen et al., 2003; Orasanu et al., 2008).

TZD induce the expression of adiponectin by adipose tissue, and adiponectin is believed to mediate some of the TZD's metabolic effects in target tissues, in particular, insulin sensitization (Nawrocki et al., 2006). Adiponectin increases fatty acids oxidation in muscles (Fruebis et al., 2001) and decreases hepatic lipid content in ob/ob mice (Xu et al., 2003). Also, adiponectin has direct anti-fibrotic and anti-inflammatory properties (Yokota et al., 2000; Kamada et al., 2003).

Administration of both atorvastatin and pioglitazone produced significant additive effects on liver enzymes, insulin resistance, liver lipids and TNF- α . This work further supports the opinion that insulin resistance and hyperlipidemia play a key role in etiopathogenesis of steatohepatitis since marked elevation of insulin resistance was associated with elevated liver enzymes and liver lipids together with inflammatory markers and when

insulin resistance was decreased by pioglitazone, this lead to marked improvement of all other parameters. On the other hand, atorvastatin failed to improve insulin resistance but could efficiently decrease hepatic lipids, serum transaminases and inflammatory markers as TNF- α , together with improvement of histopathology of liver steatohepatitis. Hence, we recommend the use of pioglitazone if the prominent feature in NASH is insulin resistance while use atorvastatin if the prominent features is hyperlipidemia and both drugs simultaneously if there are both hyperlipidemia and insulin resistance. Further studies are recommended to test for the safety of both drugs and to calculate the minimum effective dose of both drugs.

Abbreviations

NASH: nonalcoholic steatohepatitis; HFD: high fat diet; ALT: alanine transaminase; AST: aspartate transaminase; ALP: alkaline phosphatase; TZD: thiazolidinedione; TGs: triglycerides; TNF- α : tumor necrosis factor alpha.

References

- [1] Adams LA and Angulo P (2006). Treatment of non-alcoholic fatty liver disease. *Postgraduate Medical J*; 82: 315-322.
- [2] Angulo P, Keach JC, Batts KP, Lindor KD (1999). Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology*; 30: 1356-1362.
- [3] Blighard EG and Dyer MG (1995). A rapid method of total lipid extractions and purification. *Can J Biochem Physiol*; 27 (8): 911-7.
- [4] Boden G (1997). Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes*; 46: 3-10.
- [5] Bonora E, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere MB, Monauni T, Muggeo M (2000). Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care*; 23 (1): 57-63.
- [6] Browning JD and Horton JD (2004). Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest*; 114: 147-152.
- [7] Bugianesi E, Manzini P, D'Antico S, Vanni E, Longo F, Leone N, Massarenti P, Piga A, Marchesini G, Rizzetto M (2004). Relative contribution of iron burden, HFE mutations and insulin resistance to fibrosis in non-alcoholic fatty liver. *Hepatology*; 39: 179: 187.
- [8] Chitturi S and Farrell GC (2001). Etiopathogenesis of non-alcoholic steatohepatitis. *Semin Liver Dis*; 21: 27-41.
- [9] Cohen B, Noick D, Rubinstein M (1996). Modulation of insulin activities by leptin. *Science*; 274: 1185-1188.
- [10] Comar KM, Sterling RK (2006). Review article drug therapy for non-alcoholic fatty liver disease. *Aliment Pharmacol Ther*; 23: 207-15.
- [11] Da Silva Morais A, Lebrun V, Abarca-Quinones J, Brichard S, Hue L, Guigas B, Viollet B, Leclercq IA (2009). Prevention of steatohepatitis by pioglitazone: implication of adiponectin-dependent inhibition of SREBP-1c and inflammation *Hepatology*; 50 (3): 489-500.
- [12] Day CP and James O (1998). Steatohepatitis: Atale of two hits? *Gastroenterology*; 114: 842-845.
- [13] Fan JG, Zhong L, Xu ZJ, Tia LY, Ding XD, Li MS, Wang GL (2003). Effects of low calorie diet on steatohepatitis in rats with obesity and hyperlipidemia. *World J Gastroenterol*; 9 (9): 2045-2049.
- [14] Fossati P and Prencipe I (1980). Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin Chem*; 28: 2077.
- [15] Fruebis J, Tsao TS, Javarschi S, Ebbets-Reed D, Erickson MR, Yen FT, et al. (2001). Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid

- oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci USA*; 98: 2005-2010.
- [16] Gianluca SP, Cinzia C, Stefania S, Giabnna F, Tiziana B, Marco M, Samuele DM, Liliiana N, Renata S, Alessia O, Deborah B, Soren S, Antonio B, Alessndro C (2006). A model of insulin resistance and nonalcoholic steatohepatitis in rats: Role of peroxisome proliferators activated receptor- α and n-3 polyunsaturated fatty acid treatment on liver injury. *Am J Pathol*; 169 (3): 846-860.
- [17] Gomez-Dominguez E, Gisbert JP, Moreno-Monteaugudo JA et al., (2006). A pilot study of atorvastatin treatment in dyslipidemia, nonalcoholic fatty liver patients. *Aliment Pharmacol Ther*; 23: 1643-7.
- [18] Grip O, Janciauskiene S, Lindgren S (2002). Atorvastatin activates PPAR gamma and attenuates the inflammatory response in human monocytes. *Inflamm Res*; 51: 58-62.
- [19] Hotamisligil GS, Peraldi SP, Budaari A, Ellis R, White MF, Speegelman BM (1996). IRS-1 mediated inhibition of insulin receptor tyrosine kinase activity in TNF- α and obesity induced insulin resistance. *Science*; 271: 665-668.
- [20] Hyogo H, Tazuma S, Arihiro K, Iwamoto K, Nabeshima Y, Inoue M, Ishitobi T, Nonaka M, Chayama K (2008). Efficacy of atorvastatin for the treatment of nonalcoholic steatohepatitis with dyslipidemia. *Metabolism*; 57 (12): 1711-8.
- [21] Imeryuz N, Tahan V, Sonsuz A et al. (2007). Iron preloading aggravates nutritional steatohepatitis in rats by increasing apoptotic cell death. *J Hepatol*; 47: 851-859.
- [22] Ishikawa E, Hashida S, Taanaka K, Kohno T (1989). Development and application of ultra-sensitive enzyme immunoassays for antigens and antibodies. *Clin Chem Acta*; 185: 223-30.
- [23] Itaya K and Kadowaki T (1969). An improved method for colorimetric determination of free fatty acids. *Clin Chim Acta*; 26: 401.
- [24] Kabel AM, Abdel-Rahman MN, El-Sisi Ael-D, Haleem MS, Ezzat NM, El Rashidy MA (2013). Effect of atorvastatin and methotrexate on solid Ehrlich tumor. *Eur J Pharmacol*; 713 (1-3): 47-53.
- [25] Kallwitz ER, Mclachlan A, Cotler S (2008). Role of peroxisome proliferator-activated receptors in the pathogenesis and treatment of nonalcoholic fatty liver disease. *World J Gastroenterol*; 14: 22-28.
- [26] Kamada Y, Tamura S, Kiso S, Matsumoto H, Saji Y, Yoshida Y, et al (2003). Enhanced carbon tetrachloride-induced liver fibrosis in mice lacking adiponectin. *Gastroenterology*; 125: 1796-1807.
- [27] Kawaguchi K, Sakaida I, Tsuchiya M, Omori K, Takami T, Okita K (2004). Pioglitazone prevents hepatic steatosis, fibrosis and-altered lesion in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet. *Biochem and Biophys Res Comm*; 315:187-195.
- [28] Koruk M, Taysi S, Saas MC, Yilmaz O, Akcay F, Karakok M (2004). Oxidative stress and enzymatic antioxidant status in patients with non-alcoholic steatohepatitis. *Ann Clin Lab Sci Winter*; 34 (1): 57-62.
- [29] Leclercq IA, Farrell GC, Sempoux C, Dela Pena A, Hormans Y (2004). Curcumin inhibits NF-kappa B activation and reduces the severity of experimental steatohepatitis in mice. *J Hepatol*; 41: 926-934.
- [30] Lieber CS, Leo MA, Mak KM, Xu Y, Cao Q, Ren C, Ponomarenko A, DeCarli LM (2004). Model of nonalcoholic steatohepatitis. *Am J Clin Nutr*; 79 (3): 350-351.
- [31] Lowry OH, Rosebrough N, Farr AL et al. (1951). Protein measurement with folin phenol reagent. *J Biol Chem*; 193: 265-275.
- [32] Maddux BA, Sbraccia P, Kumakura (1995). Membrane glycoprotein PC1 and insulin resistance in non insulin dependent diabetes mellitus. *Nature*; 373: 448-451.
- [33] Metha K, Vanthiel DH, Shah N et al.,(2002). Nonalcoholic fatty liver disease; pathogenesis and the role of antioxidants. *Nutr Rev*; 60: 289-293.
- [34] Miyazaki Y, Mahankali A, Wajcberg E, Bajaj M, Mandarino LJ, DeFronzo RA (2004). Effect of pioglitazone on circulating adipocytokine levels and insulin sensitivity in type 2 diabetic patients. *J Clin endocrinol Metab*; 89: 4312-4319.
- [35] Nawrocki AR, Rajala MW, Tomas E, Pajvani UB, Saha AK, Trumbauer ME, et al., (2006). Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to peroxisome proliferator-activated receptor gamma agonists. *J Biol Chem*; 281: 2654-2660.
- [36] Ohkawa H, Ohishi N and Yagi K (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*; 95: 351-358.
- [37] Orasanu G, Ziouzenkova O, Devchand PR, Nehra V, Hamdy O, Horton ES, et al. (2008). The peroxisome proliferator-activated receptor gamma agonist pioglitazone represses inflammation in a peroxisome proliferator-activated receptor-alpha-dependent manner in vitro and in vivo in mice. *J Am Coll Cardiol*; 52: 869-881.
- [38] Paradise V, Perlemuter G, Bonovoust F, et al., (2001). High glucose and hyperinsulinemia stimulate connective tissue growth factor expression: a potential mechanism involved in progression to fibrosis in non-alcoholic steatohepatitis. *Hepatology*; 34: 738-44.
- [39] Ping X, Zhang X, Li Y, Yu C, Xu L, Xu GY (2006). Research on the protection effect of pioglitazone for non-alcoholic fatty liver disease (NAFLD) in rats. *J Zhejiang Uni Sci B*; 7(8): 627-633.
- [40] Reitman S and Frankel S (1957). A colorimetric method for determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. *Am J Clin Pathol*; 28: 56-63.
- [41] Richmoud W (1973): Preparations and properties of a cholesterol oxidase from *Nocardia* sp. And its application to the enzymatic assay of total cholesterol in serum. *Chin Chem*; 19 (12): 1350-6
- [42] Robertson G, Leclercq I, Farrell GC (2001). Nonalcoholic steatosis and steatohepatitis. II. Cytochrome P-450 enzymes and oxidative stress. *Am J Physiol: Gastrointest Liver Physiol*; 281: 1135-1139.
- [43] Sanyal AJ, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Shiffman MKL, Clore JN (2001). Non-alcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterol*; 120: 1183-1192.
- [44] Seki S, Kitada T, Sakaguchi H (2005). Clinicopathological significance of oxidative cellular damage in non-alcoholic fatty liver diseases. *Hepatol Res*; 33 (2): 132-4.
- [45] Song XY, Gu M, Jin WW, Klinman DM, Wahl SM (1998). Plasmid DNA encoding transforming growth factor-beta1 suppresses chronic disease in a streptococcal cell wall induced arthritis model. *J Clin Invest*; 101 (12) 2615:21.
- [46] Stepaniacke JA, Gould KE, Sun D, Swanborg RH (1995). A comparative study of experimental antiimmune encephalomyelitis in Lewis and DA rats. *J Immunol*; 155(5): 2762-9.
- [47] Tahan V, Eren F, Avsar E et al. (2007). Rosiglitazone attenuates liver inflammation in a rat model of nonalcoholic steatohepatitis. *Dig Dis Sci*; 52: 3465-3472.
- [48] Takaki A, Kawai D, Yamamoto K (2014). Molecular Mechanisms and New Treatment Strategies for non-Alcoholic Steatohepatitis (NASH). *Int J Mol Sci*; 15: 7352-7379.
- [49] Tamura Y, Kawamori R (2006). Insulin resistance. *Nippon Rhinsho*; 64(6):1071-4.
- [50] Teranishi T, Ohara T, Maeda K, Zenibayashi M, Kouyama K, Hirota Y, et al. (2007). Effects of pioglitazone and metformin on intracellular lipid content in liver and skeletal muscle of individuals with type 2 diabetes mellitus. *Metabolism*; 56:1418-1424.
- [51] Torres DM and Harrison SA (2008). Steatohepatitis: a tale of two hits? *Gastroenterology*; 114: 842-845.
- [52] Trinder P (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann Clin Biochem*; 6: 24-25.
- [53] Von Knethen A and Brune B (2003). PPAR gamma-an important regulator of monocyte/macrophage function. *Arch Immunol Ther Exp (Warsz)*; 51: 219-226.
- [54] Xu A, Wang Y, Keshaw H, Xu LY, Lam KS, Cooper GJ (2003). The fat derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. *J Clin Invest*; 112: 91-100.
- [55] Xu P, Zhang X, Li Y, Yu L, Xu G (2006). Research on the protection of pioglitazone for non-alcoholic fatty liver disease (NAFLD) in rats. *Journal of Zhejiang university Science B*; 7 (8): 627-633.
- [56] Yokohama S, Yomeda M, Hameda M et al., (2004). Therapeutic efficacy of an angiotensin II receptors antagonist in patients with non-alcoholic steatohepatitis. *Hepatology*; 40: 1222-5.
- [57] Yokota T, Oritani K, Takahashi I, Ishikawa J, Matsuyama A, Ouchi N, et al (2000). Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood*; 96: 1723-1732.