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ARTICLE

All-Trans Retinoic Acid (atRA) effectively improves liver steatosis in a rabbit model of high fat induced liver steatosis

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ABSTRACT

The aim of this study is to evaluate the role of All-Trans Retinoic Acid, the biologically active metabolite of retinoids, on liver steatosis in a rabbit model of high fat induced lever steatosis. 30 male rabbits were evaluated in 5 groups: group 1 treated with normal diet, group 2–5 included rabbit's groups 2 to 5 were fed on high cholesterol diet, group 2 received no drugs, group 3 received atorvastatin, group 4 received atRA, and group 5 received both the drugs. the liver was obtained for histopathological evaluation. oral administration of atRA, atorvastatin or their combination significantly decreased serum levels of total cholesterol, LDL, AST and ALT. atorvastatin slightly but atRA remarkably decreased liver steatosis where the difference was significant. atRA group showed the highest TAC and the lowest PCO concentrations. atRA can be effective in reducing liver steatosis and its antioxidant effect plays a crucial role in the process.

HIGHLIGHTS

- Non-alcoholic fatty liver disease (NAFLD) is the most common disorder of the liver in general population and is strongly associated with metabolic risk factors including hyperlipidaemia, obesity and diabetes.
- atRA is very effective in reducing liver steatosis and its antioxidant effect plays a crucial role in the process.
- we suggest focussing on other aspects of liver steatosis such as carbohydrate metabolism and insulin resistance in order to find better ways of controlling and treating liver steatosis.

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KEYWORDS
atRA; steatosis; antioxidant effect

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common disorder of the liver in general population (Kwak et al. 2015) that includes a hepatic steatosis, progressing to non-alcoholic steatohepatitis (NASH) and NASH with fibrosis and cirrhosis. Advancement of the disease to the stage of fibrosis and cirrhosis leads to the increased risk of hepatic carcinoma (Byrne and Targher 2020).

NAFLD is strongly associated with metabolic risk factors including hyperlipidaemia, obesity and diabetes mellitus (DM) and its management is based on decreasing these risk factors (Chalasani et al. 2012). Beside life style modifications such as diets and weight loss, various medications including orlistat (an enteric lipase inhibitor), insulin sensitising agents including metformin and thiazolidinediones and vitamin E are prescribed for NAFLD treatment (Vernon et al. 2011). But still there are some controversies on their efficacy, as studies have shown no benefits of these drugs in NAFLD management (Sanyal et al. 2010).

Retinoids are known to be involved in lipid metabolism. Retinoic acid (RA) plays significant role in adipogenesis, lipogenesis, and oxidation of fatty acid. It is also seen to reduce obesity and ameliorate insulin sensitivity. RA regulates the expression of several genes that are involved in lipid metabolism such as; stearoyl-CoA desaturase 1, UCP1, UCP3 genes and gene for medium-chain acyl-CoA dehydrogenase (Bonet et al. 2012).

All-trans Retinoic acid (atRA) is derived from vitamin A through an enzymatic process (Pellegrini et al. 2015). It binds to retinoid X receptors and RA receptors, and leads to the transcription of several genes, mentioned above (Bonet et al. 2012). It has anti-inflammatory and antioxidant activities and is involved in cellular development, growth and differentiation (Chambon 1996). atRA is also involved in cancer development, fibrosis or repair processes within liver tissue (Yanagitani et al. 2004). It is shown that knockout mice with Retinoic acid receptors (RARs) deficiency present microvesicular liver steatosis and decreased β-oxidation activity of fatty acids (Bjelakovic et al. 2010). The role of atRA in pancreatic insulin producing cells development and its regulatory role in major metabolic conditions such as obesity and DM have also been introduced (Muto et al. 1996).

As there are still controversies in NAFLD management and considering the regulatory role of atRA in obesity and DM –two main risk factors for liver steatosis- and also its

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effects on lipid metabolism (Haukeland et al. 2009) we tried to evaluate the role of atRA –the most biologically active metabolite of retinoids- on liver steatosis in a rabbit model of high fat induced lever steatosis.

Material and method

Animals

Thirty male New Zealand rabbits (aged 10 weeks, weighing approximately 2kgs) were included in the present study. All animals were housed for one week in temperature controlled (22 ± 2 °C) vivarium with relative humidity (30–60%) and day/night cycle (10 h/14h).

All animal procedures were in accordance with Guide for the Care and Use of Laboratory Animals (NIH US publication no. 85–23 revised 1985). All experiments were performed in agreement with the ethical considerations, recommended by the Pasteur Institute of Iran and the study protocol was reviewed and approved by the Ethical Committee of the Urmia University of Medical Sciences.

Drug administration

The rabbits were randomly divided into five groups. Each of these groups contained six rabbits. Group 1 was served as normal group and treated with normal diet (standard rabbit food) for 75 days. The animals in groups 2 to 5 were fed on high cholesterol diet (1% of body weight, standard rabbit food 1% cholesterol from lyophilised egg) for 75 days. Meanwhile, group 2 was served as steatosis group (positive control group) and treated with no drugs, group 3 received atorvastatin orally (20 mg/kg/day) from day 45, group 4 received atRA (5 mg/kg/day, orally) from day 45, and group 5 received both atorvastatin (10 mg/kg/day) and atRA (2.5 mg/kg/day) from day 45.

At the end of the experiment, the rabbits were fasted for 10–12 h prior to anaesthesia and peripheral blood samples were obtained from the marginal vein of rabbits' ear. Then serum samples were separated and analysed for total cholesterol (TC), triglyceride (TG), High Density Lipoprotein (HDL) and Low-Density Lipoprotein (LDL), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and alkaline phosphatase (ALP). Finally, the rabbits were sacrificed, and the liver of each rabbit was carefully dissected out.

Biochemical measurement

Blood samples were centrifuged at 3000 rpm for 10 min and serum samples were separated. Serum levels of TC, TG, LDL, HDL, AST, ALT and ALP were measured using an autoanalyzer (BT 4500, Biotechnica instruments, Italy).

Histological analysis

The liver was excised and totally immersed in phosphate buffered formalin (PBF) for at least 24 h for fixation. After fixation the samples were taken and embedded in paraffin. Then 5 μm sections were obtained from each paraffin block and stained by Haematoxylin and Eosin (H&E) method.

Oxidative stress markers

Protein carbonyl (PCO)

Protein carbonyl assessment was performed as described previously (20). This assessment is based on formation of protein hydrazone by DNPH reaction. Briefly, about 0.3 g of examined tissue was homogenised in 50 mM phosphate buffer solution and the mix was centrifuged at 4 °c by 10000 g for 10 min. after one-hour incubation, 0.5 ml trichloroacetic acid (TCA) was added. Then the mix was again centrifuged at 10000 g. The supernatant was removed, and the remnant was solved in guanidine chloride. Finally, the sample OD was obtained in 370 nm. Protein carbonyl was expressed as nmol/mg protein.

Total antioxidant capacity (TCA)

The method is performed as previously described (21). For this purpose, about 50–100 mg of the examined tissue was lyzed in KCL. After homogenisation and centrifugation, the TAC was calculated by a colorimetric method based on ferric reducing-antioxidant power (FRAP). The values were obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration and the results were expressed as mmol/L.

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Statistical evaluation of data was performed using analysis of variances (ANOVA). Normality of data was evaluated with the Kolmogorov–Smirnov test. p Values $<.05$ were considered to be statistically significant.

Results

Biochemical measurements

Lipid profile

LDL, TG and TC levels were significantly increased in high fat diet (HFD) group compared to normal control group ($p < .001$). Our data showed that oral administration of atRA or atorvastatin or their combination significantly decreased serum levels of TC and LDL ($p < .001$).

Liver function tests

Serum AST and ALT levels were significantly increased in fat diet group compared to normal control group ($p = .04$ and .02, respectively). Our data showed that oral administration of atRA, atorvastatin or their combination therapy improved

liver function tests and significantly decreased serum levels of AST and ALT, Tables 1 and 2. There was no difference between serum levels of AST and ALT in atRA, atorvastatin or their combination therapy receiving groups ($p > .05$). ALP level was not different in any of the examined groups.

Histopathologic analysis

The paraffin embedded liver samples of each rabbit were evaluated using haematoxylin and eosin (H&E) staining method. Fat diet group showed marked steatosis involving almost entire examined tissue (more than 90% of the evaluated samples), Figure 1. Oral administration of atRA, atorvastatin and their combination therapy significantly decreased the amount of liver steatosis (Figures 2–4), Table 3. Atorvastatin slightly improved liver steatosis but atRA was significantly more effective than atorvastatin and remarkably decreased liver steatosis ($p = .029$). The amounts of liver steatosis in rats receiving atRA was significantly lower than those receiving atorvastatin (Table 3). No difference was found between atRA, and their combination therapy in decreasing liver steatosis ($p > .05$).

Oxidative stress markers

Total antioxidant capacity (TAC)

TAC assay analysis showed that atRA group had the highest and atorvastatin group had the lowest TAC concentrations ($p < .001$), (Figure 5).

Protein carbonyl (PCO)

PCO assay results showed that atRA group had the lowest PCO levels and atorvastatin group had the highest PCO levels ($p < .001$), (Figure 6).

Discussion

Non-alcoholic fatty liver disease (NAFLD) is one of the most common problems around the world and is now known as the most common cause of impaired liver function and also elevated serum levels of liver enzymes (Harrison et al. 2009). It is proposed that by 2020 NAFLD would be the main cause of liver transplantation (Higuera-de la Tijera and Servín-Caamaño 2015). Excess energy uptake and diets with high carbohydrate and fat are main causes of liver steatosis.(Jiang et al. 2008) According to latest guidelines the current

Table 1. Liver function tests: positive control group that did not receive any treatment showed significantly elevated liver enzymes.

	Group 1 (negative control)	Group 2 (positive control)	Group 3 (atorvastatin)	Group 4 (atRA)	Group 5 (atRA + atorvastatin)
AST	41.4 ± 7	184.3 ± 67.8*	52.6 ± 10.8	71.4 ± 15.3	63.5 ± 10.1
ALT	48.4 ± 6.5	130.8 ± 58.6*	85.7 ± 18.1	54 ± 4.7	101 ± 20.4
ALP	119.5 ± 33	157.3 ± 21.8	162 ± 47	119.3 ± 33.2	85.6 ± 24.1

*p Values <.05.

Table 2. Comparing serum AST and ALT levels in examined groups.

AST		p Value	ALT		p Value
Group 2 (positive control)	Group 3 (atorvastatin)		Group 2 (positive control)	Group 3 (atorvastatin)	
184.3 ± 67.8	52.6 ± 10.8	.04	130.8 ± 58.6	85.7 ± 18.1	.02
Group 2 (positive control)	Group 4 (atRA)		Group 2 (positive control)	Group 4 (atRA)	
184.3 ± 67.8	71.4 ± 15.3	.03	130.8 ± 58.6	54 ± 4.7	.013
Group 2 (positive control)	Group 5 (atRA + atorvastatin)		Group 2 (positive control)	Group 5 (atRA + atorvastatin)	
184.3 ± 67.8	63.5 ± 10.1	.04	130.8 ± 58.6	71 ± 20.4	.01

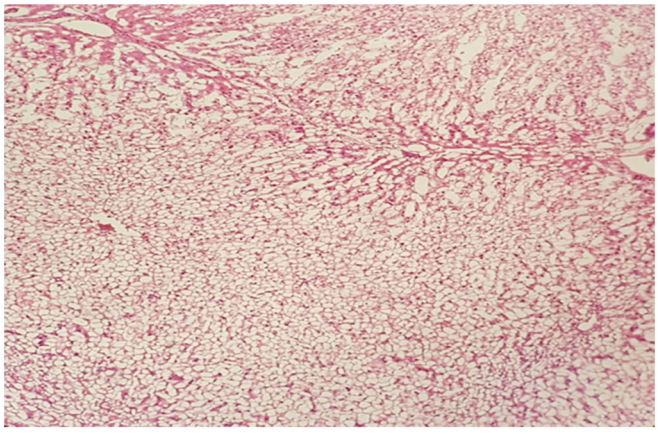

Figure 1. showing liver tissue of rabbit treated with high fat diet without any medication. There is marked accumulation of fat in liver cells which is seen in almost entire tissue (H&E, 10×).

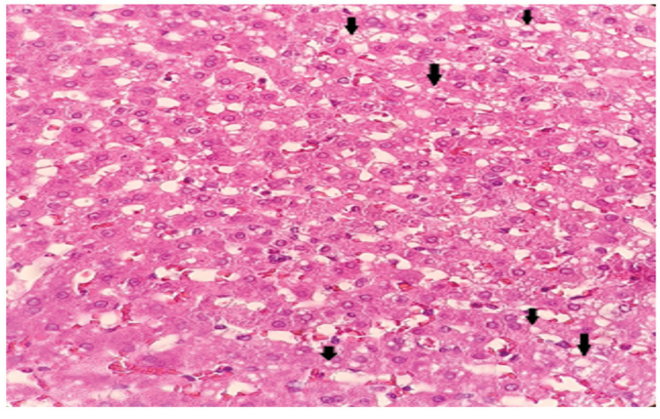

Figure 2. showing liver tissue of rabbit receiving both high fat diet and atRA. There is mild focal micro- and macrovesicular steatosis in some hepatocytes (showed by arrows). The picture shows obvious improvement in liver steatosis comparing to Figure 1. (H&E, 20×).

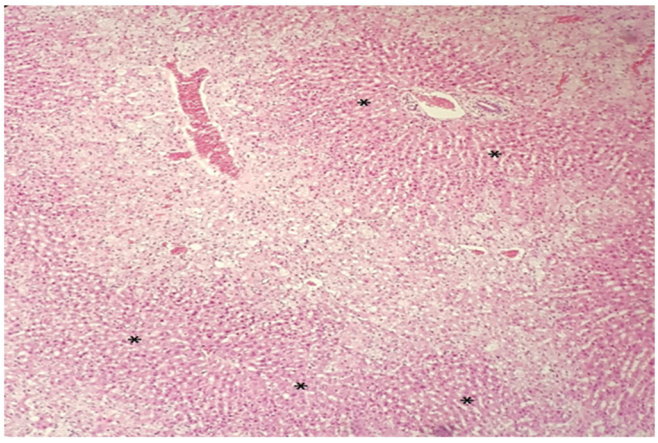

Figure 3. showing liver tissue of rabbit receiving both high fat diet and atorvastatin. There is marked steatosis in more than half of the liver specimen. Picture shows no evidence of steatosis around portal tract (area showed by *). Picture also shows mild improvement in liver steatosis comparing to Figure 1. (H&E, 10×).

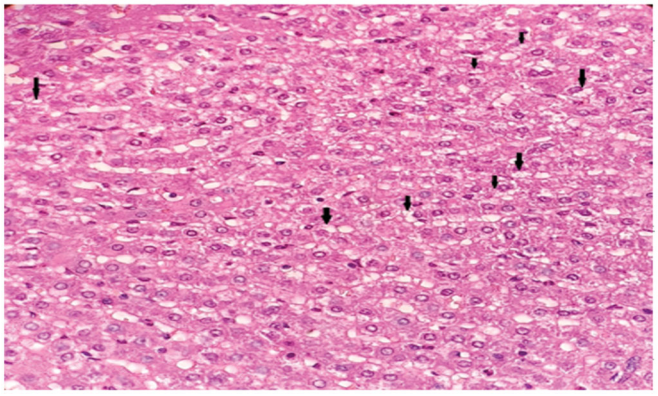

Figure 4. showing liver tissue of rabbit receiving high fat diet with both atRA and atorvastatin. There is mild focal micro- and macrovesicular steatosis in some hepatocytes (showed by arrows). The picture shows obvious improvement in liver steatosis comparing to figure 1. (H&E, 20×). atRA reduced steatosis significantly more than atorvastatin ($p = .029$).

Table 3. Comparing the amount of fatty change (steatosis) in examined liver tissue of each group.

Amount of steatosis (percent ± standard error of mean (SEM))		*p* Value
Group 2 (positive control)	Group 3 (atorvastatin)	
92.5 ± 1.4	66.25 ± 2.4	.001*
Group 2 (positive control)	Group 4 (atRA)	
92.5 ± 1.4	32.5 ± 7.7	.003*
Group 2 (positive control)	Group 5 (atRA + atorvastatin)	
92.5 ± 1.4	45.5 ± 1.4	.003*
Group 4 (atRA)	Group 3 (atorvastatin)	
32.5 ± 7.7	66.25 ± 2.4	.029*
Group 5 (atRA + atorvastatin)	Group 3 (atorvastatin)	
47.5 ± 1.4	66.25 ± 2.4	.03*
Group 4 (atRA)	Group 5 (atRA + atorvastatin)	
32.5 ± 7.7	45.5 ± 1.4	.1

*p Values <.05 is significant.

medications used for NAFLD treatment are metformin (Benzie and Strain 1996), vitamin E, Ursodeoxycholic acid (UDCA) and Thiazolidinediones (Chalasani et al. 2012). According to controversies on efficacy of the mentioned medications and considering the burden of liver steatosis search for new medications is of great interest (Zhu et al. 2016).

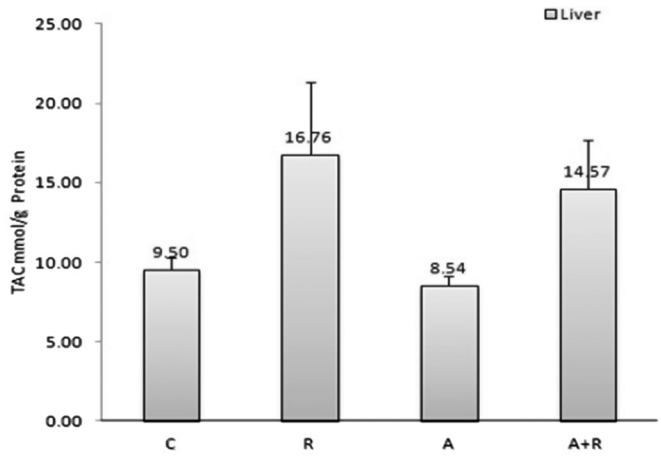

Figure 5. showing Total antioxidant capacity (TAC) assay. The figure shows that atRA group has the highest and atorvastatin group has significantly lowest TAC concentrations ($p < .001$). C: positive control; R: retinoic acid; A: atorvastatin.

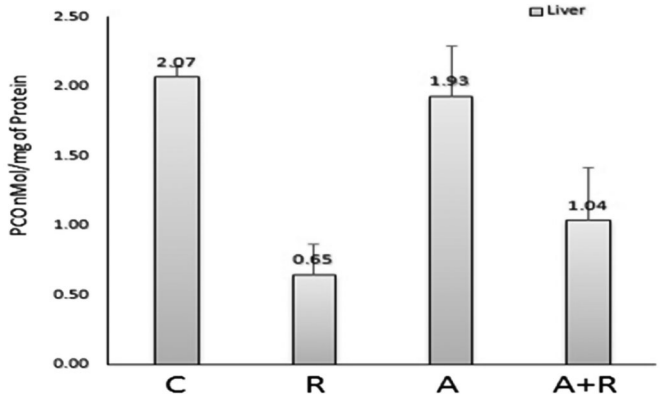

Figure 6. showing protein carbonyl (PCO) assay. The figure shows that atRA group has significantly lowest and atorvastatin group has the highest PCO levels ($p < .001$). C: positive control; R: retinoic acid; A: atorvastatin.

In this study we evaluated the effect of two drugs on liver steatosis including atRA (as a new medication) and atorvastatin- the most common drug used to decrease serum lipids- in a rabbit model of high fat induced liver steatosis. Our data showed that atRA as well as atorvastatin effectively reduced liver steatosis and we found atRA more effective than atorvastatin in this process. We also found that atRA significantly reduced liver oxidative agents (protein carbonyl) and improved serum total antioxidant capacity (TAC). According to significant improvement of liver steatosis in atRA receiving group and considering the antioxidant effects we observed in our study we assumed that antioxidant effect of atRA plays an important role in reducing liver steatosis. Antioxidant effect is mentioned as the main mechanism through which vitamin E- one of the first line pharmacotherapies for NAFLD and NASH- can reduce liver steatosis (Trasino et al. 2015). Additionally, as we found in our study, it was shown that atRA can reduce serum lipids (Pan et al. 2014). This property along with antioxidant effect can be assumed as responsible mechanisms through which atRA can reduce liver steatosis (Zhou et al. 2012).

Atorvastatin is an inhibitor of β-Hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase that converts HMG-CoA to

mevalonate, reducing the production of cholesterol by liver, increasing cholesterol clearance and increasing the expression of LDL-receptors of hepatocytes (Cioboată et al. 2017).

atRA is a vitamin A derivative which is involved in multiple cellular pathways and recently, its beneficial effects are interesting topics among researchers (Rébé et al. 2009). Various studies have performed to explore the underlying mechanism of atRA effects (Brun et al. 2013). It has been shown previously that atRA can reduce lipotoxicity- induced oxidative stress, inhibit the production of ROS (Molina-Jijón et al. 2011), and enhance fatty acid oxidation (Amengual et al. 2008). The effect of atRA on liver steatosis was also studied previously by Kim et al. (2014). They have introduced a new transcriptional cascade which can be inhibited by atRA and led to inhibition of fat accumulation in mice liver (Öström et al. 2008). Our results are in line with these studies showing that atRA can significantly improve liver steatosis.

As we induced liver steatosis using high fat diet and no antioxidant effect was observed for atorvastatin, the observed atorvastatin effect on liver steatosis in our study can be explained by its proven effect of reducing serum lipids. Although according to latest guidelines atorvastatin administration in treatment of liver steatosis is controversial but our results showed that atorvastatin can be a useful drug (although with mild efficacy) specially in patients with high serum lipids.

As a limitation, we did not explore the effect of each drug in different dosages. That was because we were not certain about their effect on liver steatosis and that is why we chose one therapeutic dose of each drug to find whether they can improve liver steatosis or not. As another limitation, we evaluated the drugs' effects in a period of 45 days. A longer treatment period may show more effective results and it can be a worthy topic of evaluation in further studies.

Totally we found that both atRA and atorvastatin can reduce liver steatosis. We showed that atRA is more effective than atorvastatin in reducing liver steatosis. The underlying mechanism of atorvastatin could be its lipid lower effect but for atRA we concluded that its antioxidant effect plays a crucial role and antioxidant effect would be an effective mechanism in reducing liver steatosis. Our data showed that atRA can be used as one of the first line pharmacotherapies for liver steatosis. Evaluating the effects of other drugs with antioxidant property on liver steatosis can be helpful in finding new medical treatments for NAFLD.

Since we found that even in high fat induced liver steatosis, anti-lipid drug showed just a little improvement, we suggest focussing on other aspects of liver steatosis such as carbohydrate metabolism and insulin resistance in order to find better ways of controlling and treating liver steatosis.

Acknowledgement

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Ethical approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Disclosure statement

The authors deny any conflict of interest in any terms or by any means during the study. All the fees provided by research centre fund and deployed accordingly

Authors contributions

Dr. Leila Zarei: Planned the study, wrote the protocol, collected the data and drafted the manuscript and accepted the final draft.

Dr.Negin Farhad: Planned and designed the study, collected the data.

Dr. Ata Abbasi: analysed the data and critically revised the draft and finally approved the manuscript.

Data availability

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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