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Are N-acetylcysteine and adalimumab effective for non-alcoholic steatohepatitis?

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Abstract

Due to the lack of effective medical treatment for non-alcoholic steatohepatitis (NASH), we aimed to evaluate new treatment options. In particular, our goal was to investigate and compare the effects of N-acetylcysteine (NAC) and Adalimumab treatment on tumor necrosis factor alpha (TNF-α) and oxidative stress during the development of NASH in a rat model of the disease. Our study included a total of 35 female Wistar albino rats that were divided into 5 groups of 7 each, and evaluated over a 6 week period. One group received a normal diet, while the other four groups received a methionine and choline deficient (MCD) diet. One of the groups receiving the MCD diet did not take any medicine, while the other three were administered NAC, adalimumab, or a NAC/adalimumab combination therapy. NASH was successfully established in the MCD diet group. Levels of TNF-α were effectively suppressed in the three groups that received therapy. Even though adalimumab significantly enhanced suppression of TNF-α, the NASH score was suppressed to a more statistically significant extent in the groups receiving NAC. Our study showed that TNF-α and oxidative stress play an important role in NASH pathogenesis. The antioxidant agent, NAC, was found to be superior to the anti-TNF agent, Adalimumab, in the improvement of total NASH score. Although these drugs did not prevent the development of NASH, it was shown that they mildly reverse the NASH histopathology score, suggesting improvement of and overall liver function.

Introduction

Non-alcoholic fatty liver diseases (NAFLD) cover a wide spectrum of liver damage ranging from NASH, to fibrosis and cirrhosis, and to steatosis.[1] NASH development during the natural course of disease is caused by inflammation, fibrosis, and cirrhosis. The pathogenesis of NASH is very complex and is typically initiated by insulin resistance. Further progressive damage to the liver is necessary to cause steatohepatitis the most severe form of NAFLD. It has been suggested that the interaction between oxidative stress and cytokines plays a key role in NASH. The increased production of Adalimumab treatment on tumor necrosis factor alpha (TNF-α) has been observed in non-alcoholic steatohepatitis (NASH) rat models. Increased serum TNF-α concentration has also been reported in humans with NASH.[2-4]

A methionine and choline deficient (MCD) diet impairs the synthesis of phosphatidylinositol choline, which is required for synthesis of very-low-density lipoprotein (VLDL). In phosphatidylinositol choline deficiency, serum triglyceride levels are decreased while liver triglyceride levels are increased. As a result, steatosis occurs.[5,6] An MCD diet displaces hepatic oxidants such as glutathione (GSH) and S-adenozilmetionin (S-AME)[7] and increases oxidative stress by suppressing oxidative defense mechanisms.[8] This cascade of events induces TNF-α and other proinflammatory cytokines[9] that contribute to NAFLD and insulin resistance with similar mechanisms. Therefore, MCD model studies are very informative because they emphasize the importance of oxidant stress in the pathogenesis of hepatic steatosis and steatohepatitis (independent of obesity). Aminotransferase levels, histological changes in the liver characterized by steatosis, focal inflammation, necrosis of hepatocytes, and fibrosis are induced in mice fed with the MCD diet. These rapid histological changes in morphology are similar to those seen in human patients with NASH.[10,11]

Lipid-lowering agents such as statins and fibrates,[12,13] antioxidants such as vitamin E,[14-16] and cytoprotective agents such as ursodeoxycholic acid (UDCA) have been shown to reduce the level of aminotransferase. In addition, they were shown to improve liver histology in several studies.[17] However, none of these drugs specifically target NASH. Therefore, there is a need for specific drugs that target and treat NASH.

Adalimumab is a human monoclonal antibody that specifically recognizes TNF-α. It has been used to treat diseases such as rheumatoid arthritis and Crohn's disease. In a previous case report, Schramm and colleagues demonstrated that the use of adalimumab for rheumatoid arthritis returned aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma glutamyl transferase (GGT) levels back to normal in patients with NASH.[9]

N-acetylcysteine (NAC) is a product of cysteine metabolism and was initialized as a mucolytic agent in the treatment of chronic lung disease in 1963. It is a precursor for the major endogenous antioxidant glutathione and has been used as an antidote in acetaminophen toxicity more than forty years. Glutathione is a tri-peptide that includes cysteine and it functions in cellular events such as amino acid transport and scavenging of free radicals, intermediate reactive oxygen products, and alkaline toxic chemicals.[18] Nakano and colleagues observed that steatotic rats had significantly lower levels of reduced glutathione in their liver when compared to non-steatotic rats. The same study showed increasing levels of reduced glutathione in steatotic rats after NAC administration.[19] NAC is also known for its function related to microcirculatory relaxation.[20] In steatotic liver, the sinusoidal space was reported to decrease by 54.0% when compared to the normal liver, and hepatic blood flow was shown to be limited to 1/3 of normal.[21] Currently, there are no effective options for medical treatment for NASH. Since TNF-α and oxidative stress play an important role in NASH, we aimed to investigate and compare the effects of NAC and TNF-α and oxidative stress during the development of NASH in a rat model of the disease.

Materials and Methods

This study was approved by the Dokuz Eylul University Ethics Committee on April 11, 2012

Animals

A total of 35 female Wistar albino rats weighing between 194-250 g (average 223.6 g)

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were obtained from the Experimental Research Laboratory, Faculty of Medicine, Dokuz Eylul University Hospital. Rats were put into cages and maintained according to the international animal experimentation and maintenance manual under standard laboratory conditions (temperature 23±2°C, 12-hour day/night cycle).

Diet

Rats were given either a normal diet or an MCD diet. The content and chemical structure was standardized by animal diet experts (Harlan Teklad, Madison, WI, USA). The rats were given 10 g/100 g food and 10-12 mL/100 g water daily.

Study design

Thirty-five female Wistar albino rats were divided into five equal groups of 7 rats in each group.

Group 1 (n=7, control): The control group. Rats were fed with standard food and water.

Group 2 (n=7, MCD): Received an MCD diet for 6 weeks.

Group 3 (n=7, MCD+NAC): Administered 20 mg/kg NAC per day by orogastric gavage and were fed with an MCD diet and water for 6 weeks. These doses were based on previously published findings.[22]

Group 4 (n=7, MCD+adalimumab): Received 7 mg/kg adalimumab intraperitoneally at weeks 0, 2, and 4 and were fed and MCD diet and water for 6 weeks. The dose of 7 mg/kg adalimumab was described previously.[23]

Group 5 (n=7, MCD+NAC+adalimumab): The rats in this group were administered 20 mg/kg NAC per day by orogastric gavage and

received 7 mg/kg adalimumab intraperitoneally at weeks 0, 2, and 4. Group five were fed with an MCD diet and water for 6 weeks.

Termination of the experiment and collecting samples

Six weeks after initiation of the study, the abdomen was opened under ether anesthesia and approximately 10 cc blood was drawn from the inferior vena cava. Blood samples were centrifuged at 5000 rpm for 5 minutes and stored at −80°C until analysis. Liver tissues were placed in 10.0% formalin and were sent to the pathology laboratory for histopathological examinations. Rats were sacrificed by ether inhalation.

Cytokine levels

TNF-α (Invitrogen, KRC3011, USA), TGF, IL-6 (Boster, EK0514, China) and IL-8 (Cusabio Int, CSB-E07273r, China) levels were measured by ELISA.

Biochemical analysis

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, total protein, and albumin levels were assessed by using Abbott Architect 16000 autoanalyzer (Abbott Inc., Princeton, NJ, USA).

Histopathologic evaluation

Liver tissue samples were obtained and fixed in paraffin blocks for processing using traditional methods for histological examination. The liver was cut for hematoxylin-eosin staining. The prevalence of fibrosis was determined by using Mason Trichrome. An expert

pathologist examined the samples under a light microscope. The presence or absence of steatosis, ballooning degeneration, lobular inflammation, and fibrosis were assessed and Brunt NASH scoring was performed. Scores of five and above were considered diagnostic for NASH.

Statistics

SPSS 15.0 computer software (SPSS Inc., Sostware Chicago, IL, USA) was used for statistical analysis. Differences between the groups were determined by Kruskal-Wallis and Mann-Whitney U tests and P-values of less than 0.05 were considered statistically significant.

Results

Rats from each group were weighed weekly starting from the first day of study. The group that was fed a normal rat diet had significant weight gain. The four groups receiving MCD diet showed weight loss, with the highest weight loss occurring in the group that received MCD diet only. Average weights of the groups according to weeks are depicted in Figure 1. No side effect was observed in any of the rats throughout the study period.

Immunological parameters

Mean serum levels of TNF-α, TGF, IL6, and IL8 in all groups are presented in Table 1. Parameters showing statistically significant differences between groups were determined with Kruskal-Wallis test. Those that were found to be statistically significant were com-

Table 1. Cytokine levels.

Immunological parameters	All rats (n=35); mean (SD)	Normal diet (n=7); mean (SD)	MCD (n=7); mean (SD)	MCD+NAC (n=7); mean (SD)	MCD+ADA (n=7); mean (SD)	MCD+NAC+ADA (n=7); mean (SD)	P*
TNF α (pg/mL)	3.80 (3.71)	1.70 (2.59)	8.60 (3.53)	4.87 (3.97)	1.82 (0.60)	2.02 (1.15)	0.002
TGF β (pg/mL)	29.60 (15.14)	15.87 (3.80)	50.93 (13.92)	27.87 (8.70)	29.63 (11.82)	23.68 (8.90)	<0.001
IL6 (pg/mL)	301.88 (123.90)	157.10 (19.75)	412.60 (84.85)	382.02 (118.50)	314.56 (110.50)	243.09 (51.65)	<0.001
IL8 (pg/mL)	147.14 (107.39)	86.96 (69.24)	179.10 (145.88)	187.01 (127.92)	142.04 (68.44)	140.60 (104.29)	0.381

*Kruskal-Wallis Test. MCD, Methionine choline deficient; ADA, Adalimumab; NAC, N-acetylcysteine.

Table 2. Statistical data for cytokine levels among groups.

Groups	TNF alpha		TGF beta		IL6	
	Z	P*	Z	P*	Z	P*
Normal Diet vs MCD	-2.875	0.004	-3.130	0.002	-3.130	0.002
MCD vs MCD+NAC	-1.663	0.096	-2.686	0.007	-0.831	0.406
MCD vs M MCD+ADA	-2.747	0.006	-2.435	0.015	-1.663	0.96
MCD vs M MCD+NAC+ADA	-2.747	0.006	-2.814	0.005	-3.130	0.002
MCD+NAC vs M MCD+ADA	-1.981	0.048	-0.128	0.898	-1.214	0.225
MCD+NAC vs M MCD+NAC+ADA	-1.853	0.064	-1.214	0.225	-2.364	0.018
MCD+ADA vs M MCD+NAC+ADA	-0.064	0.949	-1.151	0.250	-1.469	0.142

*Mann-Whitney U Test. MCD, Methionine choline deficient; ADA, Adalimumab; NAC, N-acetylcysteine.

pared between the groups using Mann-Whitney U test (Table 2).

TNF-α levels

TNF-α levels were determined as 0.99 (0.13-7.42) pg/mL in the group receiving normal diet, 9.90 (2.23-11.64) pg/mL in the group receiving MCD diet only, 3.75 (1.97-13.14) pg/mL in the group receiving MCD+NAC, 1.90 (1.09-2.58) pg/mL in the group receiving MCD+adalimumab, and 1.33 (1.12-4.24) pg/mL in the group receiving MCD+NAC+adalimumab, respectively. TNF-α levels were highest in group 2, and lowest in group 1.

Biochemical parameters

Mean serum levels of AST, ALT, ALP, total bilirubin, total protein and albumin from all groups are presented in Table 3. Parameters showing statistically significant difference between groups were determined with Kruskal-Wallis test. Those that were found to be statistically significant were compared

between the groups by using Mann-Whitney U test (Table 4).

Histopathology

Hepatic steatosis, lobular inflammation, hepatocyte ballooning, fibrosis and total NASH scores for all groups are presented in Table 5. Parameters that show statistically significant difference between the groups were determined with Kruskal Wallis test. Since the group receiving normal diet had a 0 NASH score, this parameter was not taken into evaluation with Kruskal Wallis test. Hepatocyte ballooning and total NASH score showed statistically significant difference, and were compared between groups using Mann Whitney U test (Table 6). As expected, development of NASH was histopathologically confirmed in the group receiving MCD diet. Total score of 5 or above was accepted as NASH. When total NASH scores were evaluated among the groups, the highest total score was in the

group receiving MCD+ADA with 6.71±1.06, while the lowest score was observed in the group receiving MCD+ADA+NAC with 5.00±1.15. There were statistically significant differences between the groups with respect to total NASH score (P=0.032). When MCD+NAC and MCD+ADA groups were compared for improvement in total NASH score, there was statistically significant difference in favor of MCD+NAC group (P=0.037). When MCD+NAC+ADA and MCD+ADA groups were compared, there was statistically significant difference in favor of MCD+NAC+ADA group (P=0.007). However, when the group receiving MCD diet only was compared to other groups receiving treatment, there was no statistically significant difference with respect to improvement in NASH histopathological score. As for hepatocyte ballooning, the highest score was in the group receiving MCD with 1.71±0.76; and the lowest score was in the group receiving MCD+ADA+NAC with 0.71±0.49. There

Table 3. Biochemical parameters.

Biochemical parameters	All rats (n=35); mean (SD)	Normal diet (n=7); mean (SD)	MCD (n=7); mean (SD)	MCD+NAC (n=7); mean (SD)	MCD+ADA (n=7); mean (SD)	MCD+NAC+ADA (n=7); mean (SD)	P*
AST (U/L)	123 (40.93)	134.14 (66.16)	139.86 (42.54)	115.72 (32.67)	112.71 (14.28)	112.57 (35.27)	0.594
ALT (U/L)	56.77 (20.29)	44.71 (19.68)	62.71 (29.75)	58 (14.25)	63.57 (12.23)	54.86 (20.90)	0.314
ALP (U/L)	392.89 (206.60)	472.43 (337.30)	429.29 (137.69)	468.57 (212.43)	314.29 (122.10)	279.86 (106.64)	0.283
Total Bilirubin (mg/dL)	0.40 (0.10)	0.28 (0.02)	0.41 (0.10)	0.42 (0.12)	0.43 (0.09)	0.46 (0.10)	0.005
Total Protein (gr/dL)	6.71 (0.61)	7.6 (0.57)	6.67 (0.38)	6.36 (0.36)	6.5 (0.37)	6.44 (0.47)	0.002
Albumin (gr/dL)	2.99 (0.49)	3.69 (0.43)	2.79 (0.46)	2.80 (0.30)	2.79 (0.27)	2.89 (0.25)	0.006

*Kruskal-Wallis Test. MCD, Methionine choline deficient; NAC, N-acetylcysteine; ADA, Adalimumab.

Table 4. Statistical data for the biochemical parameters among the groups.

Groups	Total Bilirubin		T. protein		Albumin	
	Z	P*	Z	P*	Z	P*
Normal Diet vs MCD	-2.564	0.010	-2.830	0.005	-2.628	0.009
MCD vs MCD+NAC	0.000	1.000	-1.489	0.136	-0.780	0.435
MCD vs MCD+ADA	-0.319	0.749	-0.712	0.476	-0.581	0.561
MCD vs MCD+NAC+ADA	-0.448	0.654	-1.160	0.246	-0.966	0.334
MCD+NAC vs MCD+ADA	-0.193	0.847	-0.837	0.403	-0.517	0.605
MCD+NAC vs MCD+NAC+ADA	-0.704	0.482	-0.193	0.847	0.771	0.441
MCD+ADA vs MCD+NAC+ADA	-0.512	0.609	-0.321	0.748	-0.853	0.393

*Mann-Whitney U Test. MCD, Methionine choline deficient; NAC, N-acetylcysteine; ADA, Adalimumab.

Table 5. Histopathological scores.

Histopathological scores	All rats (n=35); mean (SD)	Normal diet (n=7); mean (SD)	MCD (n=7); mean (SD)	MCD+NAC (n=7); mean (SD)	MCD+ADA (n=7); mean (SD)	MCD+NAC+ADA (n=7); mean (SD)	P**
Steatosis	2.06 (1.19)	0 (0)	2.57 (0.79)	2.43 (0.79)	2.86 (0.38)	2.29 (0.95)	0.567
Lobular inflammation	1.69 (1.02)	0 (0)	2 (0.81)	1.57 (0.79)	2.57 (0.53)	2 (0.58)	0.096
Hepatocyte ballooning	1.14 (0.73)	0 (0)	1.71 (0.76)	1.57 (0.53)	1.29 (0.49)	0.71 (0.49)	0.029
Fibrosis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1.000
Total NASH score	4.86 (2.30)	0 (0)	6.14 (1.07)	5.57 (0.98)	6.71 (0.76)	5 (1.15)	0.032

**Kruskal-Wallis Test (Normal diet group is not included in the analysis). MCD, Methionine choline deficient; NAC, N-acetylcysteine; ADA, Adalimumab.

was a statistically significant difference between the groups (P=0.015). As for steatosis and lobular inflammation, there was no statistically significant difference between the groups. Histopathological findings are shown in Figure 2.

Discussion and Conclusions

In this study, the NASH model was successfully achieved with administration of MCD diet. At the end of six weeks, TNF-α levels were significantly higher statistically in the second group, indicating that levels of TNF-α increased in the NASH-induced group.

When serum TGF β and IL 6 levels were evaluated, there was a statistically significant difference between the groups receiving normal diet and MCD only, which had a higher association with the second group. This showed us that TGF β and IL 6 levels increased in NASH-induced group. As for IL 8 levels, it increased more than 2 fold in the group receiving MCD diet when compared to the group receiving normal diet; however, there was no statistically significant difference between the two groups. When biochemical parameters were evaluated, there were elevations in AST and ALT levels in the group receiving MCD compared to the group receiving normal diet. However, there was no statistically significant difference. ALP level was higher in the group receiving normal diet compared to the group receiving MCD diet, which was not statistically significant. Total bilirubin, total protein, and albumin levels were significantly higher statistically in the group receiving MCD diet.

During histopathological examination, the total NASH score was found to be zero in the group that received normal diet. NASH development was histopathologically confirmed in the MCD group. Fibrosis was not observed in the NASH-induced group. Similar to previous studies, we showed that MCD diet increased proinflammatory cytokines that have been blamed responsible for the pathogenesis of NASH development, and it successfully induced NASH development. In a previous study with rat NASH models, NASH was established by giving 100.0% high-fat diet for 6 weeks. Thong-Ngam and colleagues showed that 20 mg/kg/day peroral administration of NAC significantly increased levels of anti-inflammatory acting serum glutathione, and caused regression in steatosis and necrosis-inflammation score in rats fed with high-fat diet.[22] Baumgardner and colleagues found that administration of 2 g/kg/day of NAC decreased levels of ALT, TNF-α mRNA, and cytochrome P450 2E1 in steatosis as well as the total NASH score and portal/lobular fibrosis in NASH that was induced by total enteral polyunsaturated

fat nutrition.[24] In a study by Gülbahar and colleagues, there were no improvements in serum AST, ALT and GGT levels in 11 patients with NASH upon a 5.5 months follow-up with diet. After the diet, patients were given 1000 mg/day NAC treatment perorally. Statistically,

serum AST, ALT, and GGT levels were determined to regress significantly.[25] In our study, TNF-α was found to be suppressed in the group receiving NAC when serum TNF-α levels were compared between the group receiving MCD diet and the group receiving MCD+NAC.

Table 6. Statistical data for the biochemical parameters among the groups.

Groups	Hepatocyte ballooning		Total NASH score	
	Z	P*	Z	P*
MCD vs MCD+NAC	-0.286	0.775	-0.876	0.381
MCD vs MCD+ADA	-1.164	0.244	-1.214	0.225
MCD vs MCD+NAC+ADA	-2.422	0.015	-1.615	0.106
MCD+NAC vs MCD+ADA	-1.041	0.298	-2.089	0.037
MCD+NAC vs MCD+NAC+ADA	-2.442	0.015	-0.876	0.381
MCD+ADA vs MCD+NAC+ADA	-1.927	0.054	-2.676	0.007

*Mann-Whitney U Test. MCD, Methionine choline deficient; NAC, N-acetylcysteine; ADA, Adalimumab.
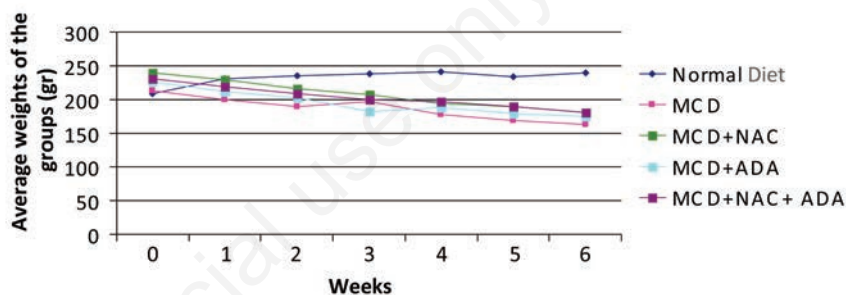

Figure 1. Average weights in the groups. MCD, Methionine and choline deficient; NAC, N-acetylcysteine; ADA, Adalimumab.
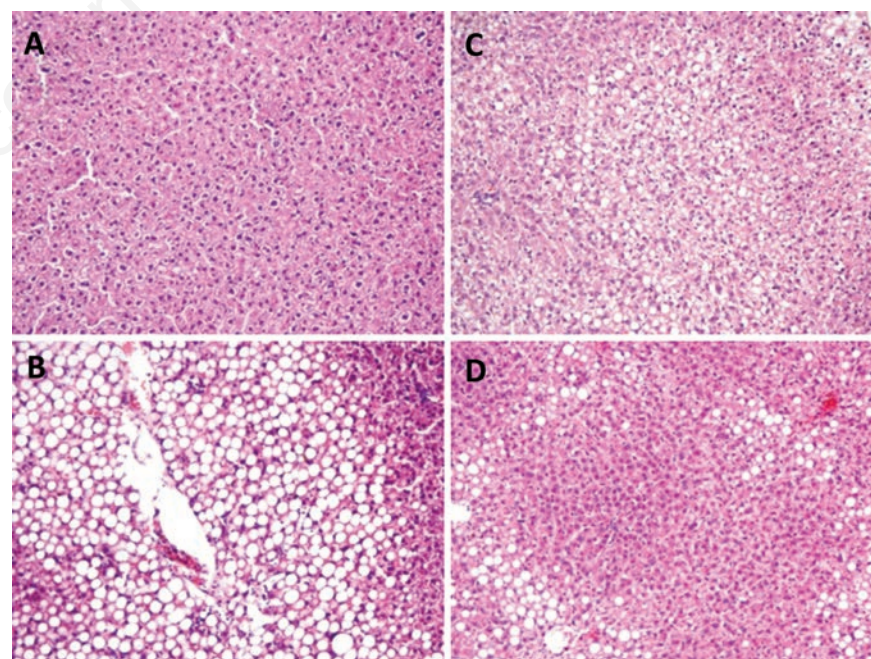

Figure 2. Normal liver tissue specimen: no steatosis, lobular inflammation or ballooning (A). Marked steatosis and lobular inflammation in the group receiving Methionine and choline deficient (MCD) diet (B). Marked hepatocyte ballooning in the group receiving MCD diet (C). Mild steatohepatitis findings in the group receiving dual treatment (D). Hematoxylin and Eosin, 200×.

However, the difference was statistically not significant. IL6 and TGFβ levels were found to be reduced in the NAC branch compared to MCD branch. The reduction in TGFβ levels was determined to be statistically significant. IL8 levels were higher compared to other groups, but the difference was not statistically significant. Histopathological examination in the group receiving NAC revealed improvement in steatosis, lobular inflammation, hepatocyte ballooning and total NASH score; however, this improvement was statistically not significant when compared with the MCD receiving group.

There were no statistically significant differences between the groups receiving MCD and MCD+NAC with respect to biochemical parameters (AST, ALT, ALP, bilirubin, albumin and total protein). There was a mild reduction in AST and ALT levels in the group receiving NAC. In their case report, Schramm and colleagues reported that AST, ALT, and GGT levels regressed back to normal in a patient with NASH after administration of adalimumab for rheumatoid arthritis.[9] Histopathological examination was not carried out in their case. In our previously published study, adalimumab suppressed TNF-α and led to improvements in liver function tests and NASH score; although, this improvement was limited. We observed slightly better results with adalimumab compared to pentoxifylline and infliximab. We concluded that suppression of TNF-α only was not sufficient in treatment of NASH, and that pathogenesis of NASH was multifactorial.[23]

In the present study, serum TNF-α levels were compared between the group receiving MCD diet and the group receiving MCD+adalimumab. Statistically, TNF-α levels were significantly suppressed in the adalimumab treated group. When the group receiving adalimumab was compared to the group receiving MCD, there was only improvement in hepatocyte ballooning score, which was not statistically significant. There was no statistically significant difference in the biochemical parameters (AST, ALT, ALP, bilirubin, albumin, and total protein) between the groups receiving MCD and MCD+ADA. However, there was slight reduction in AST and ALP levels. When compared to the group receiving MCD, there were improvements in steatosis, hepatocyte ballooning, and total NASH scores in the group receiving adalimumab+NAC; while only the improvement in hepatocyte ballooning was determined to be statistically significant. When groups receiving MCD+NAC and MCD+ADA were compared, there was greater suppression in steatosis, lobular inflammation, and total NASH score in the group receiving MCD+NAC. The suppression in total NASH score was determined to be statistically significant.

In conclusion, this present study showed that suppression of TNF-α could not prevent development of NASH, and it also did not have

a remarkable effect in the treatment. However, anti-oxidant acting NAC suppressed the total NASH score significantly and greater than adalimumab. This finding suggests that the pathogenesis of NASH is multifactorial and that TNF-α blockage has limited benefits in the treatment of NASH. Nevertheless, further studies are needed to investigate NASH pathogenesis and the benefits of different synchronous treatment options on the disease development.

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