https://doi.org/10.46389/rjd-2022-1106
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2022; volume 29, issue 3, pages 349-356

Original Article

Biochemical and biophysical changes in plasma and erythrocyte membranes of alcohol-consuming type 2 diabetics: a clinical study

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Received: 30 December 2021 / Accepted: 28 March 2022

Abstract

The effects of alcohol consumption on blood glucose levels are unpredictable , more so for an alcoholic Type 2 diabetic person. Since type 2 diabetes is a chronic condition with impairment of glucose metabolism, the influence of excess alcohol consumption in such a derailed metabolism ought to be investigated. We aimed to understand the interpolating relationship between the metabolisms of glucose and alcohol by investigating the biochemical and biophysical changes in plasma and erythrocytes, respectively. We performed a clinical study with 20 human non-alcoholic subjects, non-diabetics were considered as controls and the test subjects were categorized as alcoholics, people with diabetes and alcoholic diabetics. Findings were analyzed against the control group. Increased plasma AST, ALT, ALP, and LDH enzyme activity; higher levels of nitric oxide, thiobarbituric acid reactive species (TBARS) both in plasma and erythrocyte lysate; higher fasting and postprandial glucose, glycated hemoglobin levels (Hb1Ac); elevated levels of erythrocyte membrane total cholesterol/phospholipids (C/P) ratio and altered erythrocyte membrane fluidity in the alcoholic diabetics was noted. Alcohol induced oxidative and nitrosative stress during its metabolism and its worsening effects in people with type 2 diabetes leading to a failure in the overall metabolic homeostasis is evident from the study.

Keywords: alcohol, type 2 Diabetes, erythrocyte membrane; HB1Ac, lipid peroxidation, nitric oxide.

Introduction

Complications associated with diabetics consuming alcohol had been a point of concern globally for a very long time [1]. While people rely on alcohol consumption generally for social reasons, being an addictive drug, they generally end up becoming chronic alcoholics. The global diabetes prevalence in 2021 is estimated to be a large number, i.e., nearly 800 million, and alcohol drinking has been known to be associated with incidences of diabetes and hyperglycemic status [2]. According to previous meta-analysis studies, light-to-moderate alcohol consumption was shown to be inversely associated with the incidence of type 2 diabetes [3–5]. Health reports state diabetes and alcoholism as the second and third leading causes of global deaths [6, 7].

It is well known that alcohol affects various physiological and bio-chemical events of the human body leading to a broad spectrum of metabolic disorders [8]. Ingested alcohol readily enters the circulation exposing all the cells and tissues to a continued shock of alcohol and its metabolites for very long durations. Thus it is predictable that red blood cells and the other biochemical constituents in the circulation are significantly influenced by alcohol. Studies on the assessment

of oxidative damage on RBC membranes of alcoholic diabetics are proved to have a close relationship with associated biochemical and biophysical changes [9]. Biochemical changes in plasma and RBC membranes of diabetics and alcoholics were studied separately in human subjects [8, 10]. Nitric oxide is reported to play an important role in various physiological processes. It is also widely known that alcohol causes significant physiological damage, enhancing the burden of non-communicable diseases, and its abuse among those diagnosed with diabetes has been on a consistent rise, globally. [11]. It is generally opined that the impact of alcohol consumption on the blood glucose levels of a known diabetic is not always the same. The reasons for such an unpredictable outcome of alcohol consumption by a diabetic would probably be because of the varying carbohydrate contents of the drinks, usage of anti-diabetic drugs, their varying patterns of appetite and physical exercises they may be performing [12].

However, limited studies are available on the biochemical effects of alcohol on diabetic subjects. In the present study, we have focused on the alcohol-induced biochemical changes in some important blood constituents, such as glucose and lipids, with a special emphasis on the physico-chemical changes on the RBC membrane, which are the best model membranes used in several toxicological and membrane investigations. Hence, the present study is undertaken to investigate the same.

Material and methods

Subjects for the study

Four groups of human male volunteers, each consisting of twenty members aged between 35–60, residing in Anantapur town, Andhra Pradesh, India, were the subjects for the present study. The subjects were selected for the study based on information through a specially designed questionnaire. These volunteers were categorized into four groups as follows: controls (who were non-alcoholics and non-diabetic), alcoholics (who consumed 70–120 g of alcoholic beverage a day for the past 7–10 years), diabetics (NIDDM patients who are on metformin, glycomate medication as prescribed by the physician), alcoholic diabetics (NIDDM patients who consumed 70–120 g of alcoholic beverage a day for the past 7–10 years and also are on metformin, glycomate medication as prescribed by the physician). The beverages consumed by the chosen alcoholics included 80-proof hard liquors such as whisky, rum, Gin and brandy of various brands containing up to 40% alcohol. All the volunteers were well explained about the experimentation, written consent was obtained, and they were asked to continue with their normal regular local diet throughout the period of study. Enough care was taken to prevent the effects of diet, water or sampling time, and daily activities of the subjects. The chosen subjects were not on medication for any other known chronic diseases or illnesses and were free from using other drugs and anesthetics. Our institutional ethical committee approved the study. Baseline characteristics of the selected subjects are presented in Table 1.

Blood collection and experimentation

Venous blood samples were collected from volunteers into heparin test tubes after overnight fasting and were used for analysis immediately. Biochemical studies using plasma, erythrocyte lysate and erythrocyte membrane were carried out.

Plasma glucose and HbA1C

Plasma glucose was estimated by GOD-POD enzymatic method using a Monozyme diagnostic kit [13]. Plasma HbA1C was estimated by using ERBA diagnostic kit.

Determination of plasma and erythrocyte total nitrate and nitrite levels

As mentioned above, nitrite and nitrate levels have been determined in plasma and erythrocytes [14]. Plasma and red cell lysate samples were treated with 30% zinc sulfate to deproteinize samples followed by centrifugation at 4000 g for 5 minutes. Nitrite was measured using Griess reagent from 1.0 ml plasma aliquots and erythrocyte lysate (1% sulfanilamide, 2.5% phosphoric acid, and 0.1% 1-naphthylethylene diamine). One milliliter of supernatant aliquots was spun with cadmium granules separately for 90 minutes for nitrite conversion, and Griess was then added to the nitrate. The amounts of nitrite have been calculated using a typical sodium nitrite curve.

Estimation of plasma enzymes and lipid profile

Activities of plasma aspartate transaminase (AST; EC 2.6.1.1), alanine transaminase (ALT; EC 2.6.1.2), alkaline phosphatase (ALP; EC 3.1.3.1) and gamma-glutamyl transferase (γGT; EC 2.3.2.2) were measured.

https://doi.org/10.46389/rjd-2022-1106

Table 1: General characteristics and Haematological profile of chronic alcoholics, diabetics, alcoholic diabetics and controls.

Parameter	Controls	Alcoholics	Diabetics	Alcoholic diabetics
Age	35–60	35–60	35–60	35–60
Sex	Male	Male	Male	Male
Alcohol consumption	Do not drink	70–120 g/day	Do not drink	70–120 g/day
Chronic diseases	Not found	Not found	Not found	Not found
Diabetic history	No	No	10 years	10 years
Alchohol history	No	5 days/week	No	5 days/week
Drugs	No	No	As per physicians prescription	-
Socio-economic status	Middle and lower income	Middle and lower income	Middle and lower income	Middle and lower income
Hb (g/dl)	11.42±0.47	11.72±0.42	10.32±0.59 *	12.52±0.62 *
RBC (10^6 mm^{-3})	4.28±0.07	4.13±0.08	4.09±0.05	3.53±0.03 **
WBC (10^3 mm^{-3})	7340±167.47	8960±165.7 *	5440±37.22 **	4340±127.38 ** #
Platelets (Lacks/cumm)	181600±6915	2146600±4723 *	171330±5415 **	236600±3713 ** #
Hematocrit (%)	49.04±0.67	46.56±1.08	39.46±1.27 **	52.36±1.35 ** #
MCV (fl)	115.12±2.31	112.21±2.80	107.07±4.93 *	121.48±2.72 ** #
MCH (pg)	26.81±1.18	27.52±1.32	27.69±2.21	29.52±3.45
MCHB (g%)	23.36±1.98	25.12±2.84	26.09±1.79	29.01±3.67

Note: * Values are mean±SD of 20 human volunteers in each group. A $p<0.05$ is statistically significant betwwen groups.* – indicates significantly difference from controls; ** – indicates significantly difference from alcoholics and diabetics; # – indicates significantly different from alcoholics.

Total cholesterol (TC), phospholipids, HDL-C and triglycerides (TG) were determined using commercially available kits (Erba Mannheim, Germany) as described earlier [15]. LDL-C and VLDL-C were calculated using the formula described previously [16].

Erythrocyte membrane preparation

Erythrocyte membranes were prepared as described previously [17]. The red blood cells were lysed with 5mM phosphate buffer (PH 8.0) and spun at 15000 x g for 30 minutes after being rinsed with phosphate-buffered saline (PH 7.2). For analysis, we selected membrane ghosts that were devoided of hemoglobin after another wash with 5 mM phosphate buffer.

Determination of erythrocyte membrane TBARS

The produced malondialdehyde was used to determine the amount of lipid peroxidation (LPO) by treating the samples with 2 ml of thiobarbituric acid reagent, as reported before [17, 18].

Erythrocyte membrane total cholesterol, phospholipids and C/P ratio

Erythrocyte membrane lipids were extracted as described previously [19]. Methanol (5 ml) was added to the lysed membrane preparations, followed by the addition of chloroform (10 ml). The filtrate was removed from the mixture after 30 minutes, and the residue was utilized for another extraction. The pooled filtrates were used to estimate cholesterol [20] and phospholipids content [21].

Statistical analysis

Data were subjected to statistical analyses; values are mean±SD of 20 subjects in each group. A two-sided paired Student's t-test was performed to determine

the significant difference between the groups. A $p<0.05$ was considered statistically significant.

Results

The general characteristics and hematological profile of the controls, alcoholics, diabetics and alcoholic diabetics volunteers who participated in the study were presented in Table 1. Blood constituents, plasma, platelets, RBC and WBC were exposed to alcohol for a long time. Analysis of blood cells and their constituents provided valuable information related to the effects of alcohol and the metabolic status of the subject in alcoholic diabetes. Data presented in Table 2 revealed the information related to the changes in hematological parameters such as the concentration of Hb counts of RBC, WBC, platelets, hematocrit, MCV and MCH. Results of the study revealed that alcoholic diabetics showed a significant difference in various parameters compared to diabetics and alcoholics.

The concentrations of plasma glycated hemoglobin levels as well as glucose in both fasting and post-prandial levels in the control, alcohol, and diabetics groups were compared to alcoholic diabetics. At the same time, alcoholics and diabetics showed a significant ($p<0.05$) increase in HbA1c levels. The data presented in Figure 1 A shows that the hike in alcoholic diabetics is more pronounced than in other groups. Similarly, glucose levels were also found to be elevated in diabetics and alcoholic diabetics in both fasting and postprandial conditions and the data were presented in Figure 1 B. A not significant increase was seen in alcoholics compared to controls.

The amounts of nitrite and nitrate in plasma and erythrocyte lysate from alcoholics, diabetics and alcoholic diabetics were measured to determine NO production, and the data are presented in Figure 1 C. When alcoholic diabetics were compared to their respective control individuals, the nitrite and nitrate levels in plasma and erythrocyte lysate were significantly higher ($p<0.05$). Moreover, alcoholics and diabetics showed elevated NO levels compared to controls. The level of NO erythrocyte in alcohol diabetics has changed significantly.

Levels of total cholesterol, triglycerides and lipoprotein patterns in plasma of controls, alcoholics, diabetics and alcoholic diabetics were measured and the data are presented in Table 2. A significant ($p<0.05$) increase in plasma cholesterol, triglycerides, LDL-cholesterol, and VLDL-cholesterol, followed by a significant ($p<0.05$) decrease in HDL- cholesterol is evident from the data in alcoholics and diabetics. However, these alterations are more pronounced in alcoholic diabetics.

Alcohol intake has been linked to various metabolic alterations in the erythrocyte membrane. The oxidative stress state is determined by measuring TBARS levels. In the present study, we measured TBARS levels in control, diabetics and alcoholic diabetics and the data was presented in Figure 1 D. The erythrocyte membranes of alcoholics and diabetics had significantly higher rates of TBARS than controls. A more prominent increase was observed in erythrocyte membrane TBARS levels of alcoholic diabetics subjects than in all the other groups.

Alcohol-induced cellular damage was determined by measuring plasma enzyme levels in controls and other experimental groups; data was presented in Table 3. Alcoholics and diabetics showed elevated AST, ALT, ALP and LDH levels compared to controls. However, the hike is more pronounced in alcoholic diabetics compared to all other experimental groups.

Alcohol affects membrane fluidity, assaying total membrane cholesterol and total phospholipids gives

Table 2: Changes of plasma lipoproteins in chronic alcoholics, diabetics and alcoholic diabetics in comparison with controls.

Parameter	Controls	Alcoholics	Diabetics	Alcoholic diabetics
Total cholesterol	162.7±13.42	209.3±15.22 *	193±18.05 *	232.2±9.03 **
Troglycerides	92.7±3.12	119.3±5.26 *	153±8.05 *	202.2±9.03 ** #
HDL-Cholesterol	38.6±3.48	25.4±2.27 *	26.4±3.71 *	34.1±3.12 **
LDL-Cholesterol	21.1±1.97	19.3±0.54	41.2±3.43 *	45.1±2.67 *
VLDL-Cholesterol	15.1±1.22	13.7±1.09	19.4±1.62 *	25.2±1.42 **

Note: * Values are mean±SD of 20 samples of each group. * – indicates significantly difference from controls; ** – indicates significantly difference from alcoholics and diabetics; # – indicates significantly different from alcoholics. Values are expressed as mg/dl protein.

https://doi.org/10.46389/rjd-2022-1106

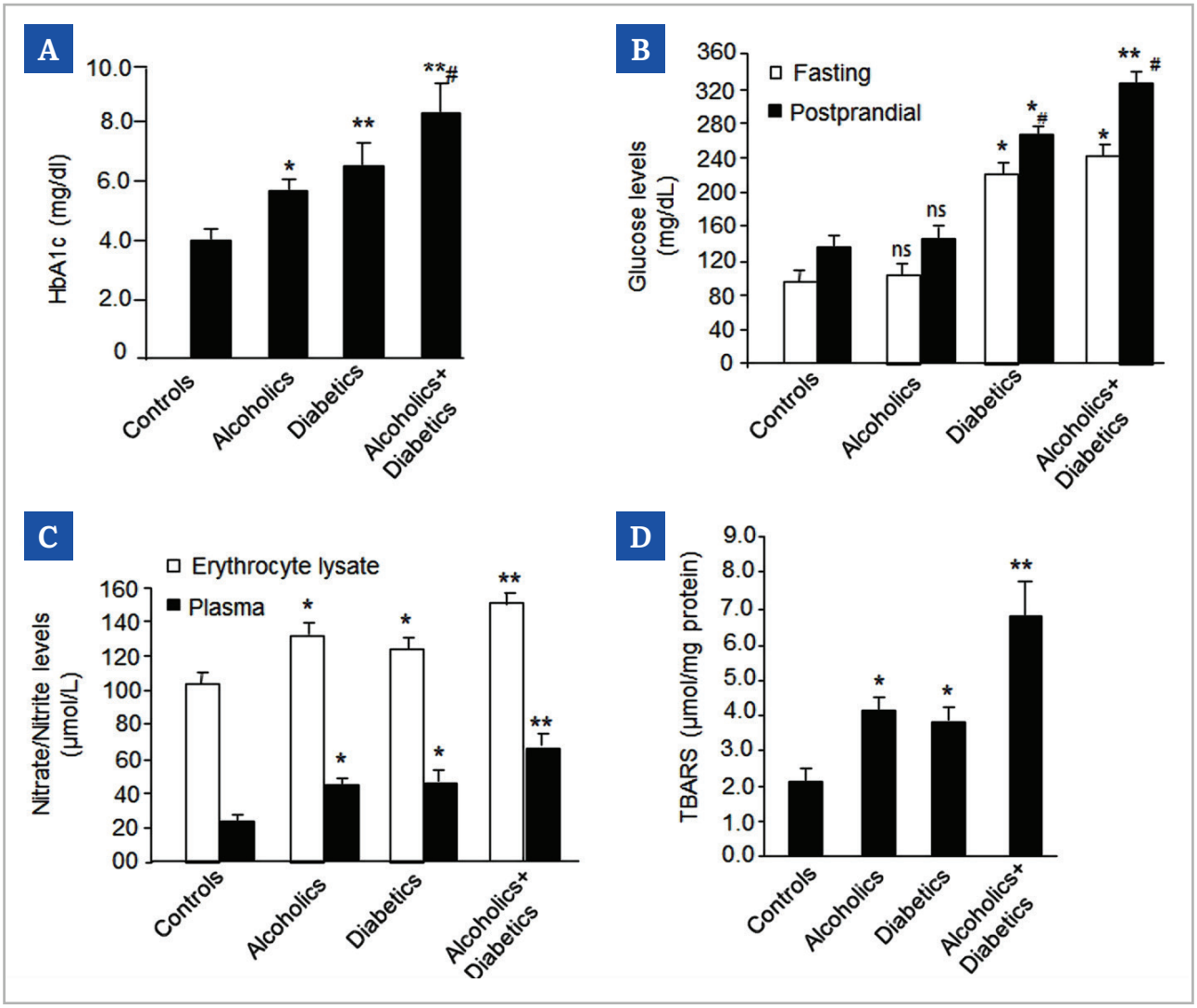

Figure 1: Effect of alcohol consumption on: A – Hb1Ac; B – glucose levels; C – nitrite and nitrate levels; and D – TBARS in Diabetics. Values are mean±SD of 20 human volunteers in each group. *– indicates a significant difference between controls; ** – indicates a significant difference between alcoholics and diabetics. # – indicates a significant difference between alcoholics. Hb1Ac and glucose levels are expressed as mg/dl. Nitrite and nitrate levels are expressed as µmol/mg protein and TBARS levels are expressed as µmol/l.

an idea about membrane fluidity. We analyzed erythrocyte membrane cholesterol and total phospholipids in control, alcoholic, diabetics and alcoholic diabetics and the data was presented in Table 4. Compared to the control group, we found a substantial rise in total cholesterol and the resulting C/P ratio in alcoholic diabetics compared to alcoholics. Moreover, alcoholic diabetics had more total cholesterol, phospholipids, and, as a result, a higher C/P ratio than alcoholics.

Discussion

Maintaining stable blood glucose levels is a finely regulated mechanism in which hormones, tissues and various other factors play a role. It is well known that hyperglycemia is a characteristic feature in diabetics due to disturbed metabolism and hormones [12]. Observed further increase in blood glucose levels of diabetics consuming alcohol in the present study suggests that alcohol provokes hyperglycemia in alcoholic diabetics. Results of the present study clearly indicates that alcohol consumption causes a continuous and prolonged increase in blood glucose levels along with a hike in protein glycation in diabetics. This is evident from the observed increase in HbA1C concentrations in alcoholic diabetics. In general, an increase in blood glucose (hyperglycemia) occurs when there is an increase in hepatic glycogenolysis and/or enhanced hepatic and renal gluconeogenesis and/or a decrease in glucose utilization by tissues. Hence liver and kidney through the former two reasons clearly seems to play a critical role

Table 3: Changes of plasma enzymes in chronic alcoholics, diabetics and alcoholic diabetics in comparison with controls.

Parameter	Controls	Alcoholics	Diabetics	Alcoholic diabetics
AST	12.71±1.01	20.32±1.43 *	19.46±1.34	29.60±1.63 *
ALT	10.39±0.83	21.56±1.29 *	19.78±1.2 *	29.80±2.08 **
ALP	65.5±8.1	92.4±11 *	97.2±5.1 *	99.6±9.5 *
LDH	13.2±0.5	17.9±1.2 *	18.3±1.5 *	21.3±1.7 *

Note: * Values are mean±SD of 20 samples of each group. All the values are expressed as IU/l. * – indicates significantly difference from controls; ** – indicates significantly difference from alcoholics and diabetics.

in the hyperglycemic condition seen in the alcoholic diabetics.

Elevated glucose levels in the system is known to cause membrane damage or cell death of red cells, cultured pericytes, kidney cells and retinal cells [1]. However, biochemical mechanisms resulting in membrane damage and cell death is yet to be known clearly. Prolonged hyperglycemia leads to the glycosylation of a number of proteins, during which the integral membrane proteins of RBCs would predominantly get glycosylated, resulting in their biophysical changes and enhanced susceptibility for degradation. The glycosylation of hemoglobin occurs by a non-enzymatic reaction between glucose and amino-terminal valine of the β-chain which rearranges to form a protein called HbA1C which is an accurate marker to diagnose prolonged diabetes mellitus [18]. Additionally, glycosylation of collagen fibers and antithrombin III also would have probably favored the acceleration of blood vessel damage that occurs in diabetic alcoholics.

Higher activities of AST, ALT, ALP and LDH in alcoholic diabetics are recorded in this study. Derangement in carbohydrate metabolism is enhanced in alcoholic diabetics, suggesting hepatic and renal dysfunction leading to increased ROS. Increased ROS may damage the membrane and cause leaking of cellular contents into the bloodstream. Increased plasma enzyme levels might be due to increased cellular membrane damage in alcoholics and diabetic alcoholics. Increased total cholesterol and plasma lipoproteins, LDL, VLDL and TG with a decrease in HDL in alcoholic diabetics suggested cardiac risk and lipid abnormalities. Circulating levels of VLDL, LDL and HDL are considered powerful indicators of cardiovascular diseases(CVD) [22]. The observed increase in total cholesterol, LDL-C, VLDL-C, and triglycerides with significantly decreased HDL-C in alcoholic diabetics compared with other groups of the present study suggested cardiovascular risk in these groups. The cardiovascular risk is as follows: alcohol diabetics>diabetics>alcohol>controls. Furthermore, this study showed increased plasma cholesterol and triglycerides with a decrease in phospholipids. Hyperlipidemia is a complication of alcohol toxicity leading to cardiovascular problems and other abnormalities. Accumulating fat in the liver is chronic alcohol intake that acts as a stimulus for the secretion of lipoprotein into the bloodstream and also the development of hyperlipidemia.

HDL is considered to be a beneficial protein that helps in scavenging cholesterol from extrahepatic tissues and the presence of lecithin cholesterol, acyltransferase (LCAT) brings it to the liver. Increased plasma cholesterol, triglycerides, VLDL, LDL and atherogenic index with a decrease in HDL concentration observed in alcoholics compared with teetotallers suggested cardiac risks as well as hepatic dysfunction in

Table 4: Erythrocyte membrane total cholesterol, total phospholipids and C/P ratio in control, diabetics and alcoholic diabetics.

Parameter	Controls	Alcoholics	Diabetics	Alcoholic diabetics
Total cholesterol	100.8±2.56	123.3±3.57 *	122.60±3.42 *	124.47±3.73 *
Total phospholipids	113.4±4.76	129.3±5.34 *	143±8.17	98.52±3.71
C/P ratio	0.88	0.88	0.85 *	1.23 **

Note: * Values are mean±SD of 20 of samples each group. Values are expressed as μg/mg protein. * – indicates significantly difference from controls; ** – indicates significantly difference from alcoholics and diabetics.

https://doi.org/10.46389/rjd-2022-1106

alcoholics. Also, in the present study, alcoholics showed increased NO production (elevated levels of nitrite and nitrate) when compared to controls. Nitric oxide (NO) mediated regulation in hepatic production or secretion of apolipoprotein particles, increasing triglycerides and lipases and decreasing the removal of circulating HDL might have played a role in the observed effects [23, 24]. Peroxidation of lipids received much attention in recent years. In the present study, the observed increase in lipid peroxidation indicates the damage of tissues and liver *in vivo*, which may cause atherosclerosis and other complications associated with alcoholism. Furthermore, increased lipid peroxidation is an indicative of enhanced oxidative stress.

The plasma nitrite and nitrate levels and the end products of NO metabolism are reliable indicators of NO production and increase concentrations of NO^2 and NO^3 in plasma of alcoholics and alcoholic diabetics. The present study suggested overproduction of NO in alcoholics. Probably this might be responsible for various abnormalities in lipid profile and the activities of enzymes in alcoholics. Earlier studies revealed alcohol-induced hepatic cytosolic NO production in rats [25]. NO Plays an important role in alcohol-induced events. Nitration of lipids and proteins is a common process under nitrosative stress. Multiple physiological actions of NO are interesting, and it interacts with molecules and free radicals in alcoholics experimentally in plasma lipids, membrane phospholipids/proteins and intracellular metabolism, playing a major role in the causation of observed alteration in lipid profile, protein profile and also in other events in cells and membranes of alcoholics, diabetics and alcoholic diabetics. Increased plasma nitrite and nitrate levels in alcoholic diabetics suggested an increased production of nitric oxide in the body, affecting several physiological activities and leading to pathological complications in alcoholic diabetics. Higher lipid peroxidation in alcoholic diabetics is recorded in this study; also, alcoholics showed increased NO production (elevated levels of nitrite and nitrate) when compared to controls. NO mediated regulation in hepatic production or secretion of apolipoprotein particles, increasing triglycerides lipases and decreasing the removal of circulating HDL might have played a role in the observed effect [23, 24].

Increased erythrocyte membrane cholesterol and phospholipids contents in alcoholics-diabetics observed in the present study indicated the transfer of cholesterol and phospholipids from plasma to the erythrocyte membrane [26]. A subsequent hike in C/P ratio suggested a decrease in erythrocyte fluidity of alcoholics in the present study. In general, alcohol perturbs the bilayer and thereby increases the fluidity of the membranes; probably, the observed reduction in membrane fluidity in the membrane of chronic diabetic alcoholics in this study can be an adaptive change leading to increased tolerance to chronic alcoholics.

Increased lipid peroxidation with an increase in plasma Nitrites and Nitrates in alcoholics strongly suggested enhanced oxidative stress with an increase in NO production leading to the generation of several free radicals. A decrease in membrane fluidity observed in alcoholics may be an adaptive biochemical change in alcoholics to counteract the fluidizing effect of alcohol. This study also strengthens and confirms the earlier reports [27] where the role of NO in alcohol-induced changes in the lipid profile of alcoholics was demonstrated.

Conclusion

In conclusion, the present study reveales that increased oxidative stress leads to pathological consequences and damage to biomembranes in alcoholic diabetics. Fluctuations in cholesterol, phospholipids and other lipid concentrations in RBC membrane with an increase in C/P ratio suggests either rigidification or fluidization of the erythrocyte membrane.

Acknowledgments

Authors thank DST-SERB for providing financial assistance and to Dr. V. Damodara Reddy under Ramanujan Fellowship (SB/S2/RJN-043/2014 dated 17.12.2015)

Conflict of interest

The authors declare no conflict of interest.

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https://doi.org/10.46389/rjd-2022-1106