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Original Research Article

Association of Serum Selenium with Oxidative Stress in Chronic Alcoholics

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Abstract: Alcoholism has caused immense morbidity and mortality to the mankind. Exposure to alcohol for a long time increases the cellular toxicity due to generation of Reactive Oxygen Species (ROS) by ethanol metabolism leading to oxidative stress, thus triggering a number of human diseases. This research emphasizes on the counter regulatory mechanisms of the body against these ROS and how these defense mechanisms, the antioxidants are interrupted by alcohol. Selenium being an antioxidant is altered by the oxidative stress in chronic alcoholics. This study was aimed to assess selenium level in alcoholic liver disease and to correlate it with oxidative stress markers like Malondialdehyde (MDA) and Gamma Glutamyl Transferase (GGT). This is a case-control study done on 48 cases of known alcoholics &48 normal healthy persons as controls. Selenium, MDA, GGT, glucose, creatinine & urea levels were estimated in all the serum samples &analyzed by student's t test &Pearson correlation coefficient .The levels of MDA & GGT in alcoholics were significantly increased while the Selenium level was significantly low in the alcoholics than the controls. Glucose levels had no significant difference. Urea & Creatinine levels were increased in cases. Increase in GGT and MDA indicates an increased risk for developing alcoholic liver diseases. The decreased selenium concentration indicates the decreased anti-oxidant status in alcoholics.
Keywords: Oxidative stress, Antioxidants, Glutathione peroxidase, Selenium, Glutathione, GGT, MDA.

INTRODUCTION

Alcohol use was the third leading risk factor contributing to the global burden of disease in 2010 after high blood pressure and tobacco smoking. According to the National Institute on Alcohol Abuse and Alcoholism[NIAAA], the Prevalence of Binge Drinking and Heavy Drinking was reported as around 24.6 percent in the age group of 18 years and above in 2011 [1]. In India, the estimated total consumption of alcoholic beverages was 200 million cases in 2009.

Alcoholism is a multifactorial phenomenon where personality, individual state of mind and social influences are in constant interaction with brain neurobiology [2]. Not only being a social disorder, it also has deleterious effects on human health , increasing the risk to many diseases and injury-related health conditions, most notably alcohol dependence, liver cirrhosis, cancers, and injuries [3,4,5]. Alcoholism or alcohol dependence is defined by the American Medical Association as "a primary, chronic disease with genetic, psychosocial, and environmental factors influencing its development and manifestations."

Liver is the main organ responsible for alcohol metabolism and occurs through two pathways-oxidative and non-oxidative. Through these pathways, ethanol produces metabolic and toxic disturbances by generation of acetaldehyde, a highly reactive and toxic that may contribute to tissue damage and possibly, the addictive process and the oxygen radicals as byproducts. Acetaldehyde is metabolized by aldehyde dehydrogenase to form acetate in mitochondria. Nicotinamide adenine dinucleotide (NAD+) is reduced during this oxidation process. Acetate is not an inert product; it increases blood flow into the liver and depresses the central nervous system, as well as affects various metabolic processes [8].

Alcohol metabolism causes changes in the NAD^+/NADH ratio in the cell, enhancing the activity of respiratory chain including heightened O2 use and ROS formation. The increased NADH/NAD ratio also raises

hepatic triglyceride concentration and causes lactic acidosis, secondary hyperuricemia & hypoglycemia. Chronic alcohol consumption has been shown to increase iron levels in the body not only when iron-rich alcoholic beverages, such as red wine, are consumed, but also because it enhances iron absorption [9, 10] and increased free iron in the cells promotes ROS generation.

Furthermore, the generated oxygen radicals lead to lipid peroxidation in cell membrane which results in the generation of additional reactive molecules like MDA and 4–hydroxy–2–nonrenal [11]. MDA and acetaldehyde reacts together with proteins to form MAAA (methoxy acetic acid adducts). Adduct formation hinders functions of normal proteins and induce harmful immune responses [12] [17].This induces a proinflammatory and profibrogenic response in liver cells [13]. Oxidative stress due to MAAA releases Cytochrome c from mitochondria which activates caspases leading to apoptosis [14]. MAAA react with several proteins like elongation factor 2 which could contribute to decline of protein synthesis. MAAA induces the activation of protein kinase, in hepatic stellate cells which in turn increases secretion of urokinase-type plasminogen activator and plays a key role in plasmin-generating system, thereby contributing to the progression of hepatic fibrosis [15]. In DNA, ROS cause strand breaks, removal of nucleotides, and a variety of modifications of the organic bases of the nucleotides. Thus alcohol has the ability to promote oxidative stress by generating free radicals and hence free radical induced tissue injury.

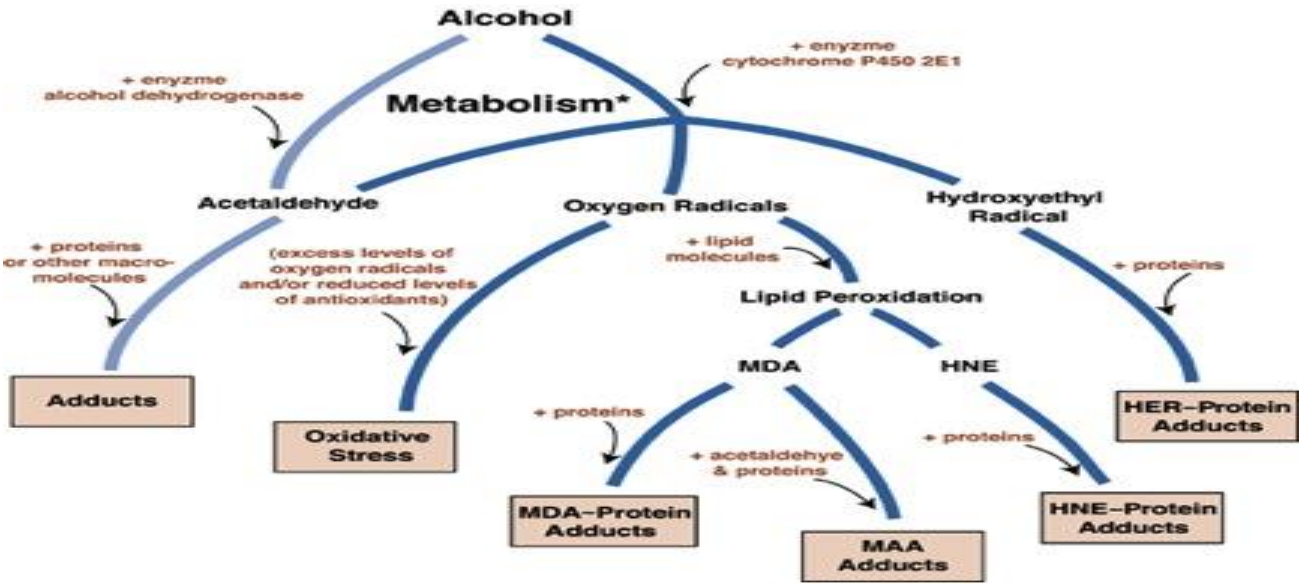

Fig 1: Dean J. Tuma, Ph.D., and Carol A. Casey, Ph.D. Dangerous Byproducts of Alcohol Breakdown—Focus on Adducts-national institute on alcohol abuse and alcoholism

Excessive production of oxygen radicals and/or a concurrent deficiency of antioxidants cause oxidative stress, which can lead to cell death. The enzymatic antioxidants involved in scavenging the free radicals include superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). SOD, one of the most effective intracellular enzymatic antioxidant catalyzes the rapid removal of superoxide radical's .The GPx system which is Selenium dependent, helps to remove hydrogen peroxide and plays a protective role in oxidative stress-induced apoptosis [16].

The GPx system includes the enzymes GPx, glutathione reductase and the cofactors glutathione (GSH) and NADPH. The various isoforms of GPx are GPx-1, the most abundant crucial antioxidant enzyme and present in all cells [17]. GPx-2, epithelial-specific enzyme that is highly expressed in intestine; GPx-3 ,a secreted subtype and GPx-4. Thus GPx plays an important role together with GSH and other cofactors like selenium which is present as seleno cysteine at its catalytic site to combat oxidative stress. Thus Selenium availability regulates GPx enzyme activity. GSH is biosynthesized by gamma-glutamyl cycle catalyzed by GGT which plays a key role in GSH homeostasis. GGT helps by breaking down extracellular GSH and providing cysteine, the rate-limiting substrate, for intracellular denovo synthesis of GSH. Thus the

oxidative stress created by free radicals is combated by the GPx enzyme with Selenium and induces increased synthesis of GSH by increasing the expression of GGT.

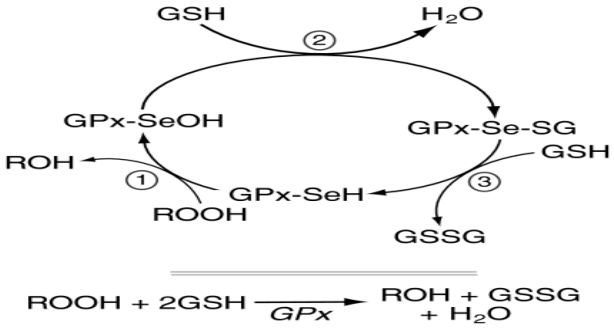

Fig 2: Reduction of H2O2 (Edith Lubos et al; Glutathione Peroxidase-1 in Health and Disease: From Molecular Mechanisms to Therapeutic Opportunities)

Selenium, a trace element is an essential constituent of a number of enzymes. Its deficiency makes animals susceptible to injury by certain types of oxidative stress [18]. It is an essential component of several major metabolic pathways [19]. Selenium is a structural component of the active center of the animal enzyme cellular GPx1 is incorporated as seleno cysteine into the active site of a growing polypeptide chain [20, 21]. In GPx, four arginine residues and a lysine residue provide an electrostatic architecture which in each reductive step directs the donor substrate GSH towards the catalytic center in such a way that its sulfhydryl group must react with the Selenium moiety [22, 32]. A unique mechanism in the regulation of GPx-1 is that of Seleno cysteine incorporation [23, 24]. In a selenium-deficient environment, cells have only about 5% of normal human GPx1 activity.

Alcoholic liver disease is associated with enhanced lipid peroxidation, protein modification, formation of hydroxyethyl radical and lipid radicals and decrease in the hepatic antioxidant defense [25]. This study is an attempt to reveal the role of selenium in preventing or ameliorating the toxic actions of alcohol [26].Further, this study tries to correlate selenium levels and oxidative stress markers like MDA and GGT. If the levels of GGT correlates with MDA and selenium concentration, then measuring GGT alone will predict the antioxidant status since assessment of MDA is time consuming and laborious.

MATERIALS & METHODOLOGY:

This case control study was conducted in the Department of Biochemistry, Tertiary care teaching hospital in TamilNadu, India during April-June 2014. The study protocol was approved by the Ethics committee of the hospital, and all subjects gave their informed consent before participation in the study.

STUDY POPULATION:

48 cases of known alcoholics and 48 normal healthy persons as controls.

CASE DEFINITION:

According to the "Dietary Guidelines for Americans 2015-2020," U.S. Department of Health and Human Services and U.S. Department of Agriculture, moderate drinking is up to 1 drink per day for women and up to 2 drinks per day for men.

Examples of one drink include:
Beer: 12 fluid ounces (355 milliliters)
Wine: 5 fluid ounces (148 milliliters)
Distilled spirits (80 proof): 1.5 fluid ounces (44 milliliters)

Heavy or "at-risk" drinking
For men: more than 4 drinks on any single day and more than 14 drinks per week
For women: more than 3 drinks on any single day and more than 7 drinks per week
Inclusion criteria- known alcoholics for more than 6 months duration with heavy or at risk drinking
Exclusion criteria - Those individuals who consume statins.

INVESTIGATIONS
Sample: 5ml of random venous blood sample, centrifuged and the serum aliquoted and stored at -20^{0}C.
Following investigations were done:
1. Blood Glucose: GOD/POD method.(Enzymatic, End point analysis)
2. Blood Urea: UV-GLDH method

3. Serum Creatinine: Modified Jaffe's Reaction

4. Estimation of Selenium:
Reference range-0.02-0.95µg/ml
Method: Spectrophotometric determination of selenium
Reagents: Azure B, potassium iodide, hydrochloric acid ,
NaHSeO3-standard

A standard stock solution of selenium was prepared by dissolving 1.910g of NaHSeO3 in 1000ml of water Standard concentrations of 0.25,0.5,1.0,2.0,4.0µg/ml were prepared. The standard graph was obtained.

Reagents: 2M Hydrochloric acid, 2% potassium iodide solutions. 0.1% solution of azure B in methanol-water mixture .

Procedure: 100 microliters of sample was mixed with 50µL of 2% KI and 50 µL of 2M HCl. This mixture is briefly shaken until a yellow colour develops. To this 50µL of azure b is added and shaken for two minutes. It is diluted with 650ml of distilled water and is read at 630nm against Reagent Blank which is prepared as above by adding 100µl of distilled water in place of sample.

5. Estimation of MDA:
Reference range-0.19-0.22µmol/L
Method: BEUJE JA & AUSTIN JD METHOD

Reagents: Thiobarbituric acid (TBA), trichloroacetic acid (TCA), 1,1,3,3 tetra methoxy propane (used to prepare standards for MDA)
The various concentrations of MDA standards were prepared (1, 2, 4, 6 & 8 µml/L). The standard graph was obtained.
Procedure
Three test tubes were taken and marked blank, standard and test. 0.5 ml of de ionized water, standard & sample were added respectively .Then to each test tube 0.5 ml of deionized water,1ml of TCA & 1 ml of TBA are added. The test tubes were kept in a boiling water bath for 15 minutes and then ice cooled to stop the reaction. Then the contents are vortex mixed for 1 min and centrifuged at 1000 g for 10 min. The clear pale supernatant was collected and read at 532 nm. The results were expressed in µmol/L.
Principle: The MDA reacts with TBA to generate the MDA-TBA adduct with pale pink colour. The MDA-TBA adduct can be easily quantified at 532nm.

6. Estimation of GGT:
Reference value-0-50 IU/L
Method: Kinetic (modified Szasz) Method

RESULTS

The study population consisted of 96 men- 48 chronic alcoholics and 48 normal healthy controls .The study groups were matched for age, sex and risk factors. Comparison of biochemical parameters between study groups are listed in the Table 1.

Table -1: comparison of biochemical parameters between study groups

VARIABLE	GROUP	MEAN	STD DEVIATION	p value
GLUCOSE (mg/dl)	CASES	123.15	42.32	0.144
	CONTROLS	138.28	57.25	NS
UREA (mg/dl)	CASES	32.82	20.59	0.044
	CONTROLS	25.87	11.45	S
CREATININE (mg/dl)	CASES	1.34	1.05	0.003
	CONTROLS	0.86	0.25	S
GGT (IU/L)	CASES	71.26	77.13	0.000
	CONTROLS	26.24	11.26	S
MDA (µmol/L)	CASES	1.30	0.97	0.002
	CONTROLS	0.83	0.24	S
SELENIUM (µg/mL)	CASES	0.38	0.49	0.000
	CONTROLS	0.95	0.60	S

P value < 0.05 – significant
S- Significant; NS- not significant

Among the routine parameters glucose has no significant difference between the groups while urea & creatinine levels are high in the cases and statistically significant. GGT & MDA levels are found to be higher in the cases compared to the controls with statistical significance while selenium level is significantly decreased in alcoholics with p value < 0.000. There is no significant correlation between GGT and MDA,

GGT and selenium as well as between MDA & Selenium.

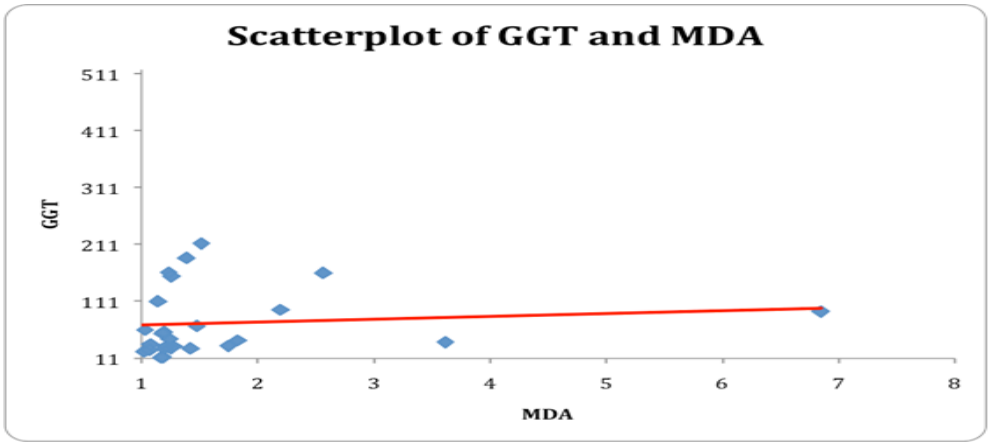

Fig-1: Scatterplot of GGT and MDA

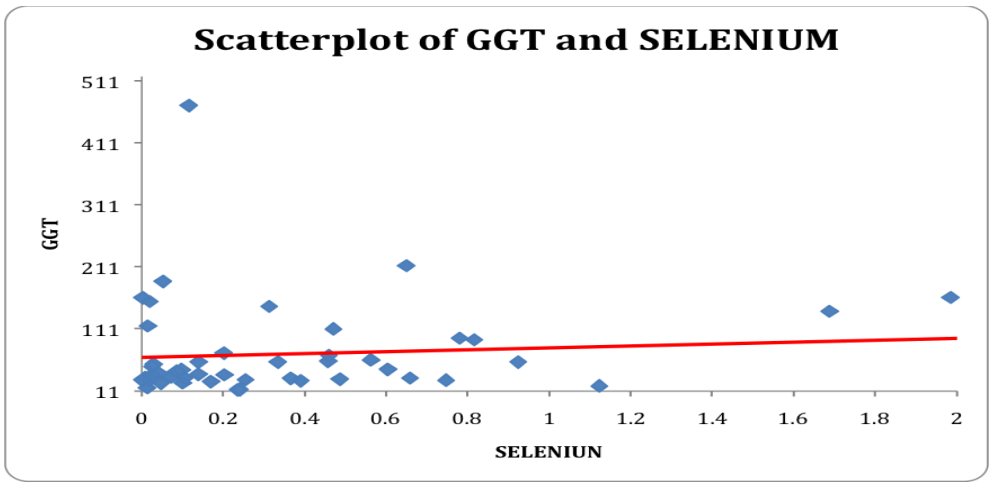

Fig-2: Scatterplot of GGT and selenium

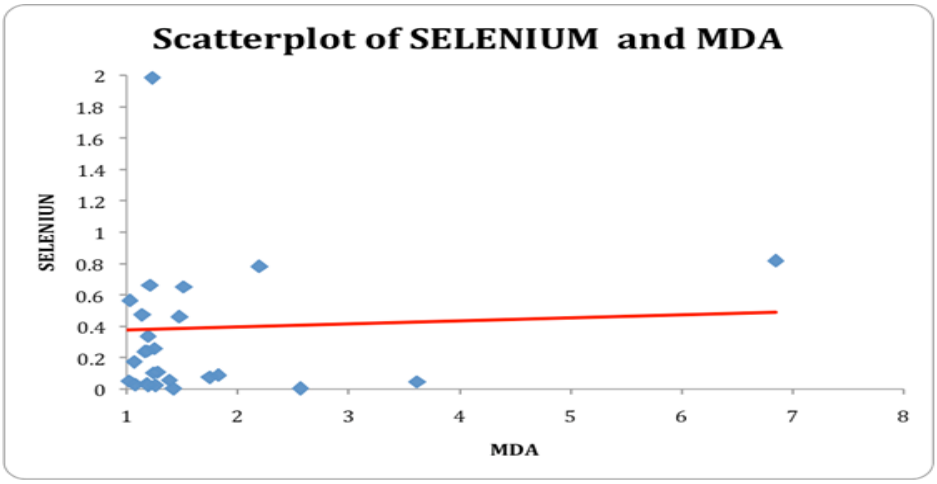

Fig-3: Scatterplot of selenium and MDA

DISCUSSION:

This study states that the oxidative stress markers GGT and MDA are increased in chronic alcoholics compared to the risk factor matched controls. There was a substantial increase in the activity of GGT in alcoholics as compared to that in the controls and the expression of GGT increases as an adaptation to oxidative stress thereby increasing the biosynthesis of GSH. This is supported by Hietala et al in their study on Serum GGT in alcoholics, moderate drinkers and abstainers. In their study, GGT values in the individuals consuming alcohol was significantly higher than those in abstainers (P <0.001)[27]. Ethanol is known to deplete GSH levels via the generation of oxidants as well as by inhibiting the mitochondrial GSH transporter [28]. Another mechanism may involve the direct conjugation of GSH with acetaldehyde [29]. Also ethanol induces hepatic GSH depletion by increasing efflux of GSH from the liver and by enhancing its utilization for the detoxification of free radicals and oxidants [30]. As an adaptation to oxidative stress, GGT gene expression is increased which helps to combat the stress [31, 32].

The increased levels of plasma MDA in the alcoholics than the controls reveal the oxidative stress prevailing in alcoholics. Our results are supported by the study of Masalkar PD *et al.;* in the study 'Oxidative stress and antioxidant status in patients with alcoholic liver disease [33]. MDA is an easily detectable biomarker of oxidative stress.

The concentration of Selenium was significantly low in the alcoholics as compared to that in the controls. This again emphasizes the fact that antioxidants are decreased in alcoholics accompanying the relatively increased oxidative stress. Tanner et al have published their study in which both serum selenium and vitamin E levels were shown to be significantly depressed in the alcoholic study groups and serum selenium was more markedly depressed in subjects with established liver disease. In chronic alcoholics the high incidence of duodenal ulcer decreases selenium absorption [34, 35]. Selenium depletion is also related to diminish dietary intake in alcoholics. Moreover alcoholic beverages are a poor source of selenium [36]. Thus Selenium deficiency increases lipid peroxidation by reducing the activity of GPx which adds to oxidative damage of liver cell membranes [37]. Selenium supplementation in alcoholic cirrhosis could improve liver function [38]. Exogenous Selenium supply has been found to control the enzymatic activity of human GPx1.

In studies by Manta singh et al GGT activity showed a significant positive correlation with MDA. However in this study such positive correlation was not observed due to small sample size and less reliable information given by the subjects which serves as the limitation of this study. There is no significant correlation between GGT and Selenium and between MDA and Selenium in this study. But the study conducted by Manta singh *et al.;* [39]. Reveal negative correlations between GGT and GSH, and between MDA and GSH. Increased levels of GGT causes increased consumption of GSH which in turn decreases selenium concentration which explains the negative correlation. However this result was not obtained in this study again may be due to small sample size. But this study clearly demonstrates that alcoholics have a compromised antioxidant defense system. Creatinine is also increased in cases compared to that of controls because of the fact that alcohol disturbs nitrogen balance in the body, increases protein metabolism and makes blood urea nitrogen level to rise and increases burdens to the kidneys. Hence clearance of creatinine is decreased [40, 41]

CONCLUSION

- GGT and MDA being the oxidative stress markers are significantly increased in alcoholics indicating the increased risk for developing alcoholic liver diseases and other complications.
- The anti-oxidant status is decreased in alcoholics as indicated by decreased Selenium and its supplementation improves the anti-oxidant defense system thus ameliorating the oxidative stress in alcoholics to prevent further complications.

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