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Biochemical Studies on Alprazolam (APZ) Users in Anantapur District of Andhra Pradesh State, India

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ABSTRACT
The purpose of this study is to evaluate Alprazolam effects on blood metabolites, Red Blood Cell (RBC) membrane and Nitric Oxide (NO) levels on long term use by human volunteers. A total of 16 subjects from Anantapur District of Andhrapradesh State were taken and divided into two groups of eight subjects each. Group I served as control (non APZ users) and group II served as test sample (long term APZ users). Biochemical studies were conducted with plasma and RBC membrane of two groups. Result of this study revealed that the concentrations of glucose, cholesterol and a lipoprotein LDL-C were decreased, and in contrast the concentrations of tri-glycerides, lipoproteins (HDL-C & VLDL-C) and nitrates were increased in plasma of group II when compared with group I. Very slight increased and decreased concentrations of HbA1c and nitrites were detected in plasma of group II, respectively. Similarly, Studies on RBC membrane were evident that significant depletion of cholesterol and phospholipids in group II.

Key words: *Alprazolam, benzodiazepine, RBC membrane, Nitric Oxide, Lipoproteins.*

1. INTRODUCTION

Stress is one of the main route causes for many diseases in human populations. It is generally considered to be immunosuppressive and to increase susceptibility to infections and cancer [1, 2]. Many anxiolytic drugs are now commercially available and widely using in treatment for control of stress and its associated disorders. One of such drug is alprazolam (APZ) and is a benzodiazepine (BZD) anti-anxiety agent frequently used for the treatment of generalized anxiety, panic attacks with or without agoraphobia and depression in humans as well as in dogs [3-5]. Almost 20% of the global populations in the world are intermittently using BDZs. Among which APZ tops the list with highest consumption [6]. APZ is easily absorbed and extensively metabolized in humans after oral administration. In order to get desirable effects APZ widely using for recreational purposes [7], and also with drinks [8a, 8b] and fruit juices [9]. Despite the record of safety of this short term acting drug, its higher consumption, long term and lavish use without prescription shows adverse effects. Many studies were conducted to know the effect of APZ on human subjects. In one such study, some long term, high-dosage users of APZ developed reversible depression [10]. The use of APZ in pregnancy is believed to be associated with congenital abnormalities. Evidence is also available that the APZ decreases the levels of female hormones such as FSH and LH in humans (authors unpublished data). Overdose symptoms may include extreme drowsiness, confusion, muscle weakness, loss of balance or coordination, feeling light-headed, and fainting, coma and death.

Though India ranks top in APZ manufacture (5.1 tons) as well as consumption [6], research contribution towards APZ is negligible. So far, no information is available on its long term use and their consequences at blood metabolites and RBC membrane. Hence, the study is undertaken with a view to evaluate the changes induced by the long term use of APZ in human volunteers for past few years.

2. MATERIALS AND METHODS

2.1. Subjects for the Study

A total of 16 male human volunteers (subjects) of 34-45 years old residing in Anantapur town of Andhrapradesh State were taken for this study. Among the all subjects, 8 were free of sedatives and any other anti-depressants or tranquilizers and were considered as group I and remaining were taken under group II who consumed Alprazolam (APZ) for the past six months to three years daily 0.25-0.5 mg twice a day (Table 1). Both the groups were free from any other chronic disease or illness,

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smoking habit and other psychoactive drugs. All the volunteers were well explained about the experimentation and their written consent was obtained.

2.2. Blood Collection

Blood samples from overnight fasted subjects were colleted, stored at -4°C in refrigerator and finally used for conducting this study. Blood was drawn from each subject (from both the groups) by vein puncture between 7 to 10 AM into heparinized test tubes and immediately chilled to 4^{o}C later plasma and red blood cells were separated for biochemical analysis.

2.2.1. Plasma Analysis

Plasma was analyzed to determine the biochemical parameters such as concentration of glucose, triglycerides, cholesterol, lipoproteins (High Density Lipids-HDL, Low Density Lipids-LDL and Very Low Density Lipids-VLDL), nitrites and nitrates.

Table 1: Characteristic Features of Subjects.

Parameter	Group	
	Group I (Control)	Group II Chronic APZ users
Number (n)	8	8
Age (years) & gender	43 ± 2 (male)	43 ± 2 (male)
APZ (mg/day)	--	0.25-0.5

2.2.1.1. Estimation of plasma glucose

Plasma glucose was estimated by using Monozyme diagnostic kit, which is based on the method of Tinder [11].

2.2.1.2. Estimation of plasma HbA1c

Using TRBA diagnostic kit (which was developed based on the method of Trivelli *et. al.,* [12]) plasma HbA1c (hemoglobin A_1 protein) was determined.

2.2.1.3. Estimation of plasma total cholesterol

Plasma total cholesterol and HDL-Cholesterol were estimated by the enzymatic kit method of Allian et.al., [13]. LDL and VLDL cholesterol were determined by the formula of Friedward *et. al.*, [14].

2.2.1.4. Estimation of tri-glycerides

Plasma tri-glycerides were estimated by using Qualigens diagnostic kit, which developed based on the adaptation of the method Fossati and Priciple [15].

2.2.1.5. Estimation of nitrites and Nitrates

Nitrite and nitrate levels in plasma were estimated as described by the method of Sastry *et. al.,* [16].

2.3 *Biochemical Analysis of Erythrocyte Membrane*

2.3.1. Isolation of erythrocytes

To isolate erythrocytes, blood was diluted with saline and then passed through a cellulose column [17]. The filtrate collected was centrifuged at 1000 rpm for 10 min. The erythrocytes settled as pellet were separated by removing the upper supernatant carefully. This washing step was repeated and the finally obtained erythrocytes were used for estimating the lipid peroxidation and preparation of erythrocyte ghosts.

2.3.2. Preparation of erythrocyte membrane (ghost preparation)

The erythrocyte membranes (or) ghosts were prepared by using the method Dodge *et. al.,* [18].

2.3.3. Membrane analysis

Total lipid was extracted from different cellular fractions and was done by the method Ways and Hanahan [19]. The phospholipids and cholesterols of the membrane were respectively estimated by Connerty *et. al.,* [20] and Zlatkis *et. al.,* [21] methods.

2.4. Osmotic Fragility of Erythrocytes

The test of osmotic fragility was attempted to determine the concentration of solute inside the red cells by placing the cells in different concentrations of NaCl i.e., 0.9 % to 0.1% and also to determine the hemolysis in hypotonic solution. The intracellular solute concentration as reflected by the red cell fragility can be helpful in establishing the pathologic state of the red cells. NaCl in the concentration range of 0.1% to 0.9% was taken in '9' different centrifuge tubes with a volume of 10 ml. 1 ml of 50 % 1:1 diluted red cells suspension was added to each tube and mixed immediately by gentle swirling, allowed to stand at room temperature for 30 min. After 30 min of incubation, tubes were remixed and centrifuged at 2000 Xg for 5 min. and absorbance of the supernatant was read at 540 nm against blank (Tube containing 0.9% NaCl with no hemolysis).

The percentage of hemolysis of the each tube is calculated as follows.

$$\text{Percentage of hemolysis} = \frac{\text{O.D. of individual tube X 100}}{\text{O.D of 100\% hemolysis (0.1\%)}}$$

STATISTICAL ANALYSIS

Data was analyzed for significant difference ($P \leq 0.05$) among values of controls and chronic APZ users using STUDENT 't' test.

3. RESULTS AND DISCUSSION

The actual mechanism underlying in the long term use of Alprazolam are not clear yet. In the present study, an attempt has been made to evaluate the

biochemical changes induced in blood and erythrocyte membrane of long term use of APZ in human volunteers. The results of this study presented in table 2 and 3. A significant decrease in the plasma glucose (92.95 Vs 79.89 mg/dl, 13%) and HbA1c (5.64 Vs 5.06, 0.5 %) concentrations were observed in group II when compared to group I. Observed decrease in plasma glucose and glycosilated Hb (HbA1c) suggesting that the glucose lowering effect by APZ. HbA1c is a precise index on long term glucose homeostasis reflecting the average blood glucose concentration. Though the plasma glucose and HbA1c are within normal range, significant reduction in the values of these parameters in APZ users clearly showed the effect and possible consequences. Maintenance of constant levels of blood glucose is complex process in which various hormones, enzymes, several tissues and other factors play a role. Blood glucose concentration is regulated chiefly by two processes viz., glycogenesis and glycogenolysis in liver [22]. In addition, blood glucose levels are regulated by hepatic and renal gluconeogenic production of glucose on one hand and degradation of peripheral glucose on the other [23].

Circulating levels of VLDL, LDL and HDL are considered to be powerful risk factors for cardiovascular diseases (CVD) [24]. A significant decrease in plasma cholesterol moiety in group II (219.5 Vs 197.78) indicated hypocholesterolemic effect of APZ in human volunteers using APZ for the past few years. Studies of Shiori *et. al.,* [25] demonstrated that significant reduction of cholesterol by APZ treatment.

Increase in plasma triglycerides (145.5 Vs 171.2), HDL-C (49.23 Vs 58.17), VLDL-C (12.42 Vs 30.63) with a decrease in LDL-C (66.46 Vs 54.72) are recorded in group II of the present study suggesting the unique action of the tri azolo-benzodiazepine, the APZ, on lipids and lipoprotein patterns where a mixed effect with a risk and benefit was observed. HDL cholesterol is considered as anti-atherogenic good cholesterol involved in the transport of sterols and lipids [26]. The concentrations of HDL-C have inverse relationship with Coronary heart disease (CHD) in humans. The low concentrations of HDL-C have associated with higher risk of CHD and rising concentrations are associated with a fall in risk of CHD [27]. Based on the epidemiological data available, the NCEP ATP III (National cholesterol education program, Adult treatment program III, 2001) [28] the HDL-C levels more than 60 mg/dl considered as high while less from 40 mg/dl to 35 mg/dl was considered as low [29]. The definition of "optimal" HDL-C will likely undergo further modification [30]. In the present study, group II have elevated levels of HDL-C, when compared with group I indicating beneficiary effect in lowering risk of CHD. In contrary, individuals with low HDL-C (<40 mg/dl in men and <50 mg/dl in women) are at increased risk of Cardio vascular disease (CVD) [31], which leads to restenosis followed by coronary balloon angioplasty [32] and cardiovascular death [33].

Hike in VLDL-C and triglyceride (TG) increases the susceptibility of the subjects for cardiovascular risk. Plasma triglycerides (145.5 Vs171.2) and VLDL-C (12.4 Vs 30.63) levels were increased significantly in APZ users and this may be due to stimulation of VLDL-TG synthesis. Delayed clearance of chylomicron-TG or VLDL-TG might have contributed for the same decrease. VLDL belongs to the common spectrum of triglyceride rich particles. Some VLDL synthesized in the small intestine, acts as a vehicle for reabsorption of endogenous cholesterol and fatty acids of biliary origin [34]. Synthesis of VLDL is promoted by an increase in influx of free fatty acids in liver and ultimately the particles are converted to LDL. The increased circulatory VLDL-C and the associated triglycerides due to defective clearance [35,36] of the particles from circulation is in agreement with earlier studies and the changes are attributed to the altered activity of lipoprotein lipase [37].

Low density lipoproteins are cholesterol rich particles containing 70% of total plasma cholesterol mainly as cholesterol esters and are derived from progressive delipidation of VLDL and serve to deliver cholesterol to liver and peripheral cells. They are bound and taken up by specific high affinity receptors, which recognize Apo-protein-B, the major Apo-protein of LDL [38] of critical importance for the regulation of plasma LDL [39]. LDL cholesterol concentrations are strongly and positively related to CHD and atherosclerotic complications [40]. LDL deposits cholesterol in artery wall, forming plaque whereas HDL removes cholesterol from plaque and form the blood stream. Therefore, increased levels of LDL and small dense LDL are associated with the risk of coronary artery disease [41]. Lower levels of LDL-C have been observed in group II when compared to group I (66.46 Vs 54.72) indicating favor the group II subjects from the risk of above said diseases in this study. However, this benefit may not be applicable to the subjects who consume APZ more than the 3 years (subjects of the present study are taken APZ from 6 months to 3 years).Adverse effects relay on the drug dose, prolonged consumption and age factors of the patients. Apart from this glycosylation induces compositional and structural changes in LDL.

When normal human erythrocytes pre-incubated with APZ (0.05 mg/ml) with different concentrations of NaCl ranging from 0.1-0.9% and extent of hemolysis was examined to assess the influence of APZ on membrane tolerance/osmotic fragility of red cells. The results of the present study was presented in the table 2 and revealed the decrease in tendency of hemolysis at various concentrations of NaCl solutions which indicating the protective effect or tolerance related to red cell membranes. Significant decrease in red cell membrane cholesterol (100.44 Vs 83.01) and phospholipids (108.18 Vs 84.90) moieties as well as the consequent C: P ratio (0.92 Vs 0.97) in APZ users indicated decrease in membrane fluidity leading alterations in physicochemical properties in APZ users (Table 3).

Table 2: Osmotic fragility in red blood cells**.**

S.No	Concentration of NaCl	Osmotic fragility	
		Control	APZ users
1	0.1	0.41	0.34
2	0.2	0.37	0.32
3	0.3	0.32	0.30
4	0.4	0.11	0.19
5	0.5	0.13	0.15
6	0.6	0.09	0.10
7	0.7	0.07	0.08
8	0.8	0.06	0.06
9	0.9	0.05	0.05

Nitric oxide (NO) is diffusible free radicals which play many roles as an effecter molecule in diverse biological systems including neurotransmitter, anti-microbial and anti- tumor activities [42]. Available literature strongly suggests that low (or) moderate increase in NO production exerts several beneficiary effects and excessive production leads to several and pathological consequences. Plasma levels of nitrite and nitrate are the reliable indicators of NO production in the body. Increase of nitrite (3.05 Vs 3.79) and nitrate (32.45 Vs 37.79) levels in plasma of group II subjects of this study indicates an increase NO production in APZ users (Table 3). NO appear to play an important role APZ induced events. Nitrosation of lipids and proteins is a common process under nitrosative stress. NO behaves as antioxidant and pro-oxidant depending on oxidative stress [43]. APZ induces to increase NO production in cell, blood and other tissues [44,45]. In view of versatile role of NO, the observed changes in plasma profile (plasma glucose, lipids, lipo-proteins -HDL-C,LDL-C,VLDL-C, Nitrite, Nitrate), membrane constituents and physicochemical properties of bio-membranes (membrane fluidity, membrane constituents) of the present study cannot be ruled out.

Table 3: Effect of long term use of Alprazolam on plasma glucose, HbA_1C cholesterol, Tri-glycerides, Lipoproteins, Nitrite, Nitrate and RBC membrane cholesterol and Phospholipids.

Parameter	Group	
	Group I (Control)	Group II (Chronic APZ users)
	Plasma	
Glucose	92.95±2.68	79.895±1.95
HbA_1C	5.642±0.21*	5.0625±0.19*
	Lipids	
Cholesterol (mg/dL)	219.50±4.25	197.78±5.20*
Triglycerides (mg/dL)	145.50±6.32	171.20 ± 5.26*
	Lipoproteins	
HDL-C (mg/dL)	49.23±2.93	58.17 ±2.46*
LDL-C (mg/dL)	66.46±3.67	54.72 ± 3.32*
VLDL-C (mg/dL)	12.42±1.84	30.63 ± 2.14*
	Nitric oxide (NO)	
Nitrite (µmoles/L)	3.05 ± 0.08	3.79 ± 0.05*
Nitrate (µmoles/L)	32.45 ± 0.11	37.62 ± 0.40*
	RBC membrane	
Cholesterol (mg/dl)	100.44 ±1.29	83.01 ± 1.17*
Phospholipids (mg/dl)	108.18 ± 1.87	84.90 ± 2.11*
C/P ratio	0.92	0.97

Values are Mean ± SEM. Plasma glucose level and plasma HbA1C concentration is expressed as mg/dL. *Values are significant from control $p \leq 0.05$.

4. CONCLUSIONS

Alprazolam, a tri-azolobenzodiazopine is a globally used potential drug with anxiolytic, hypnotic and anti-depressant actions. This benzodiazepine is generally prescribed for short term use. However, due to several reasons people have been using this drug for long term purposes. So far, no information is available on its long term use and their consequences. In the present study an attempt was made to evaluate its effects on long term use by human volunteers. Findings of this study revealed APZ induced alterations in blood glucose homeostasis, decrease in plasma glucose level as well as alterations in plasma lipid, lipoproteins. Significant decrease in red cell membrane cholesterol and phospholipid moieties as well as the consequent C: P ratio in APZ users indicated decrease in membrane fluidity leading to alterations in physicochemical properties in APZ users. Increased NO in the blood and erythrocyte membrane of APZ users is an evident for NO action. Furthermore APZ appears to be rendering tolerance to erythrocyte membrane and thereby preventing RBC hemolysis to some extent. The actual mechanism of APZ induced alterations and

in blood and erythrocyte membrane is not known and is needed to be elucidated.

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