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INTERACTION OF RED GRAPE EXTRACT AND NICOTINE IN THE LUNG TISSUE OF MALE ALBINO RAT WITH REFERENCE TO AGING

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ABSTRACT
Cigarette smoking is the main risk factor for various chronic diseases, including cardiovascular disease, pulmonary disease and cancer. Wine is a widely consumed beverage in the world, with thousands of years of tradition. The phenolic compounds in grape berries are responsible for some of the major organoleptic properties of wine, such as color, astringency, bitterness, and aroma. Age matched rats were be divided into 4 groups of six in each group and treated as follows: Group I: Normal Control (NC) (Control rats received 0.9% saline). Group II: Nicotine treated (Nt) (at a dose of 0.6 mg/ kg body weight by subcutaneous injection for a period of 2 months). Group III: Red grape extract treated (RGEt). (Red grape extract at a doses of 25 mg/ kg body weight via orogastric tube for a period of 2 months). Group IV: Nicotine + Red grape extract treated (Nt+RGEt) (The forth group of rats were received the nicotine + red grape extract as followed by the second and third group). The animals were sacrificed after 24 hours after the last treatment by cervical dislocation and isolated the lung washed with ice-cold saline, immediately immersed in liquid nitrogen and stored at - 80^0 C for biochemical analysis and enzymatic assays. In the present study the Total Carbohydrates, Glycogen and Total Free Amino Acids, were significantly decreased in nicotine treated rats in lung and increase was observed in the combination treatment (Nt+RGEt), This study suggests red grape extract treatment may be beneficial to young and old age subjects due to nicotine intoxication.

KEYWORDS: Nicotine, Red Grape Extract, Total Carbohydrates, Glycogen, Total Free Amino Acids, Lung and Male Albino Rats.

INTRODUCTION
Grape (*Vitis vinifera* L.) is one of the world's largest fruit crops (Shaker, 2006). With nearly 70 million tones currently produced worldwide grapes are possibly the world's largest cultivated fruit, most of which are used in wine making, while the rest are consumed as table grapes or processed into raisins, juices, jams or other products. Of all grapes, cultivars of the *Vitis vinifera* L. species are the most important throughout world, but especially in Europe (Mazza, 1995**)**. There are dozens of other less important species of grapes that belong to *Vitis* genus (Chalker-Scott, 1999; Zhao, *et al.*, 2010).

Comparing diets among western countries, researchers have discovered that although the French tend to eat higher levels of animal fat, surprisingly the incidence of lung disease remains low in France, a phenomenon named the French Paradox thought to occur from protective benefits of regularly consuming red wine. Apart from potential benefits of alcohol itself, including reduced platelet aggregation and vasodilation (Providencia, 2006), polyphenols (e.g., resveratrol) mainly in the grape skin provide other suspected health benefits such as(Opie and Lecour, 2007); 1) Alteration of molecular mechanisms in blood vessels reducing susceptibility to vascular damage, 2)Decreased activity of angiotensin, a systemic hormone causing blood vessel constriction that would elevate blood pressure, 3)Increased production of the vasodilator hormone, nitric oxide (endothelium-derived relaxing factor).

Nicotine is a naturally occurring alkaloid found primarily in the members of the Solanaceae family (*Nicotiana tobacco*), which includes tobacco, potato, tomato, green pepper, and eggplant. Nicotine was first isolated and determined to be the major constituent of tobacco in 1828 (Schevelbein, 1982). In commercial tobaccos, the major alkaloid is nicotine, accounting for about 95% of the total alkaloid content (Jacob *et al.*, 1993). Tobacco use is the leading cause of death in the world today. With 4.9 million tobacco-related deaths per year, no other consumer product is as dangerous or kills as many people as tobacco (WHO, An international treaty for tobacco control, 2003).

Cigarette smoke is a complex mixture of more than 4700 chemical compounds including free radicals and oxidants. Toxicity exhibited by cigarette smoke may be due to combined action of these compounds inducing many cellular processes mediated through reactive oxygen species (ROS). Major Player probably nicotine as it is present in tobacco, in higher concentrations. The compounds that induce intracellular oxidative stress recognized as the important agents involved in the damage of biological molecules. Experiments using animal and cell culture model systems suggested that moderately higher concentrations of some forms of ROS like NO and H_2O_2 can act as signal transducing agents.

Nicotine also induces oxidative stress both in *vivo* and in *vitro* that causes a peroxidant/ antioxidant imbalance in blood cells, blood plasma and tissues (Suleyman *et al*., 2002). Oxidative stress generates free radicals that attack on the membrane lipids resulting in the formation of malondialdehyde (MDA), which causes peroxidative tissue damage (Srinivasan and Pugalendi, 2000). Animals studies have shown significantly higher liver and serum levels of MDA, conjugated dienes, hydroperoxides, and free fatty acids in rats induced by cigarette smoke (Ashakumary and Vijayammal, 1996; Zhang *et al*., 2001). Smokers incur a sustained free radical load that increases their ascorbic acid (Vitamin-C) (Helen and Vijayammal, 1997) and a-tocopherol (vitamin E) (Tsuchiya *et al*., 2002) requirement. Supplementation with ascorbic acid and α-tocopherol is considered safe and ease, because these are susceptible to dietary manipulation (Byers and Perry, 1992). This study was designed to investigate the effects of Red grape extract on nicotine induced oxidative stress in the Lung tissue of male albino rat.

MATERIAL AND METHODS

Pathogen free, wistar strain male albino rats of two age groups (3 months and 18 months) 3 months age group considered as 'Young age' and 18 months age group considered as 'Old age' as per the life span of Wistar strain, (Jang *et al.*,2001) were used in the present study. The usage of animals was approved by the Institutional Animal Ethics Committee (No: 2012/2013/(i) a/CPCSEA/IACE/SVU/KC/dt. 01/07/2012). The rats were housed in clean polypropylene cages under hygienic conditions with photoperiod of 12 hours light and 12 hours dark. The rats were fed with standard laboratory chow (Hindustan Lever Ltd, Mumbai) and water *ad libitum*.

Selection of age group: In the book, entitled "International care and treatment of rabbits, mice, rats, guinea pigs and Hamsters" published by W.B. Saunders Co., Philadelphia, USA., Schuchman, (1989) given a detailed table regarding the age and life span of different strains of laboratory animals. As per this study the maximum life span of a rat is 3 years. Cao and Cutler, (1995) studied aging process from 6 months age through 12 and 24 months. Jang *et al.*, (2001) studied age related changes in 2.5, 5, 10 and 23 months of Wistar strain rats. In their study 12 months age group rats were considered as the second highest age group. Thus, the literature pertaining to selection of age group in the field of "aging" is variable in various studies.

Maintenance of animals for three years long period to attain maximum aging in the laboratory is practically difficult. The puberty of the rat reaches in between 50 – 60 days (i.e., 2 months). So, any time after 2 months is considered as "matured age". The rat attained 12 months age considered as "middle age" and after that age is the "old age". However, between 12 months and 36 months the animal becomes older and with diminished physiological functions. On the basis of the physiology of the animal, in the present study "3 months age" group considered as "Young" and "18 months age" group was considered as "Old" for effective comparison of aging process in relation to red grape extract treatment and nicotine treatment.

Dosage of Nicotine: The dose administration of nicotine was followed as per the protocol given by (Shoaib and Stolerman, 1999; Helen *et al*., 2003) 0.6 mg / kg body weight (0.5ml) was chosen as the dose, for this study.

Grouping of Animals: Age matched rats were divided into 4 groups of six in each groups. i) Control, ii) Nicotine treatment (Nt), iii) Red Grape extracts treatment (RGEt) and iv) Nicotine+ Red Grape extract treatment (Nt+RGEt).

Group I–Control: Six rats were treated with normal saline (0.9%) orally via orogastic tube for a period of 2 months.

Group II–Nicotine treatment (Nt): Rats were received the nicotine at a dose of 0.6 mg/kg body weight (0.5ml) by subcutaneous injection for a period of 2 months.

Group III – Red Grape extract treatment (RGEt)
Rats were received red grape extract 25mg/kg body weight via orogastric tube for a period of 2 months.

Group IV – Nicotine + Red Grape extract treatment (Nt+RGEt): Rats were received the nicotine at a dose of 0.6 mg/kg body weight (0.5ml) by subcutaneous injection and red grape extract 25mg/kg body weight via orogastric tube for a period of 2 months.

The animals were sacrificed after 24 hrs after the last treatment session by cervical dislocation and the lung tissue were isolated at -4^0, washed with ice-cold saline, immediately immersed in liquid nitrogen and stored at -80^0 for biochemical analysis and enzymatic assays. Before assay, the tissues were thawed, sliced and homogenized under ice-cold conditions. Selected parameters were estimated by employing standard methods.

BIOCHEMICAL ANALYSIS

1. Total Carbohydrates: The total carbohydrate content was estimated by the method of Carroll *et al.,* (1956). The lung tissue was homogenized in 10% Trichloro acetic acid (TCA) to prepare 1% (W/V) homogenates. The proteins precipitated were removed by centrifuging the homogenates for 15 minutes at 3000g at 4^0C. The clear supernatant was taken for the estimation of total carbohydrates. To 0.5 ml of supernatant, 5 ml of anthrone reagent was added and kept in a boiling water bath for 15 minutes. Then, the contents were cooled and read at 620 nm against the reagent blank. The total carbohydrate content was expressed as mg of glucose/gm wet weight of the tissue.

2. Glycogen: The Glycogen was estimated by the method of Kemp and Van Hejnigen, (1954). The lung tissues were homogenized in 80% (W/V) methanol to prepare 5% (W/V) homogenates. The suspension was centrifuged at 3000g for 15 minutes at 4^0C the supernatant containing glucose was decanted. (The glycogen content present in the lung tissue homogenates was estimated after extraction of the glucose with 80% methanol). Now the lung tissue residue was suspended in 5 ml of deproteinizing solutions (5% TCA containing 0.1% silver sulphate) and the fluid level was marked on centrifuge tube and the tube was covered with a glass cap and placed in a boiling water bath for 15 minutes. Then the tube was cooled in running tap water and deproteinizing solution was added up to the mark to compensate the loss due to evaporation. The contents were centrifuged at 5000g for 15 minutes at 4^0C. 1ml of clear supernatant was added to 3ml of concentrated sulphuric acid in a wide mouthed test tube mixed by vigorous shaking. The mixture was heated in a boiling water bath for exactly 6.5 minutes and subsequently cooled under running tap water. The intensity of the pink colour developed was read against the blank at 520 nm in a spectrophotometer. The glycogen content was expressed in mg of glucose/gram wet weight of the tissue.

3. Total Free Amino Acids

The total free amino acids were estimated by the method of Moore and Stein, (1954). 5% (W/V) homogenates of lung tissues were prepared in 10% (W/V) trichloro acetic acid (TCA) and centrifuged the contents at 2000g for 15 min at 4^0C. To 0.5 ml of supernatant, 2.0ml of Ninhydrin reagent was added and the contents were exactly boiled for 6½ minutes in a boiling water bath. The contents were cooled to laboratory temperature. The samples were made upto 10 ml with distilled water and the colour intensity was read at 570 nm in a spectrophotometer against the reagent blank. The total free amino acid content was expressed in mg of free amino acids per gram wet weight of the tissue.

STATISTICAL ANALYSIS

Statistical analysis has been carried out using INSTAT software. The data was analyzed for the significance; the results were presented with the P-value.

RESULTS AND DISCUSSION

A carbohydrate is an organic compound that consists only of carbon, hydrogen, and oxygen, usually with a hydrogen oxygen atom ratio of 2:1 (as in water); in other words, with the empirical formula $C_m(H_2O)_n$. (Some exceptions exist; for example, deoxyribose, a component of DNA, has the empirical formula $C_5H_{10}O_4$). Carbohydrates are one of the three major food groups needed for proper nutrition (proteins: 20-25%, carbohydrates: 50-60%, Fat: 20-30%). Carbohydrates in food are important and immediate source of energy for the body. Starch refers to carbohydrates found in plants (grains), vegetables and fruits are a source of starch and are broken down to sugar or glucose.

In the present study the total carbohydrates content was decreased in both age groups (young and old) of nicotine treatment rats (young by –23.00%; old by –33.96%) when compared to control rats. In red grape extract treatment rats of both (young and old) an increase (young by 3.68%; old by 5.34%) was observed than the control rats. In the combination treatment (Nt+RGEt) slightly increased was observed when compared to control rats of both age groups (Table.1, Fig.1). In the present investigation it was observed that the age induced slight elevation in total carbohydrate content in the lung, which may be due to decreased metabolic utilization in the old animals. The impaired alterations in the activities of enzymes involved in the carbohydrate metabolism contribute to the reduction of carbohydrate catabolism and elevation in age-related accumulation of tissue carbohydrates. The age-related slowing down and impairment in carbohydrate metabolism appears to play a role in the expression of cellular senescence (Tollefsbol, 1987). The decrease in total carbohydrate levels in the lung of old rats after nicotine treatment suggest possible utilization of carbohydrates to meet the energy demand during nicotine toxicity. Nicotine produces stress in the body both *in vivo in vitro* (Suleyman *et al.,* 2002). Barry and Mizock, (1995) reported stress causes to the alteration in the carbohydrate metabolism. These alterations include enhanced peripheral glucose uptake and utilization, hyperlactatemia, increased glucose production, depressed glycogenesis, glucose intolerance, and insulin resistance.

The ability to metabolize carbohydrates is reduced with advancement of age. An age related decrease in respiratory activity and metabolic utilization of carbohydrates has been observed in kidney (Sailaja, 1997; Gurumurthy, 2001), heart tissue slices (Bilwanath, 1996; Subhan *et al*., 2013). Enzymes of Kreb's-citric acid cycle show diminished activities with age (Ermini, 1972). Cartee *et al.,* (1993) reported decreased activity levels of glucose-6-phosphate dehydrogenase and glucose-6-phosphofructokinase with advancement of age. Young rats can more readily maintain high levels of oxygen consumption accompanied by a more efficient use of fats, carbohydrates as an energy source compared to old ones (Somani *et al.,* 1992). The decreased glycolytic and Kreb's-citric cycle enzymes which are necessary for the catabolic process of carbohydrates, may lead to increase the total carbohydrate content in the kidney of old age rat. We observed in the present study the induced effect of red grape extract treatment (RGEt) increase the total carbohydrates content in both age groups at the same time decrease was observed in the nicotine treatment (Nt) rats when compare to control rats. Interestingly, in the present investigation with combination treatment (Nt+RGEt) an increase in the levels of total carbohydrate content was found in the lung tissue of both age groups of rats. Thus, these results clearly suggest that, the red grape extract treatment (RGEt) was beneficial for nicotine subjects.

Glycogen is a multi branched polysaccharide that serves as a form of energy storage in animals (Sadava *et al*., 2011). In humans, glycogen is made and stored primarily in the cells of the liver and the muscles, and functions as the secondary long-term energy storage (with the primary energy stores being fats held in adipose tissue). The amount of glycogen present in tissues varied widely with diet and physiological status (Nelson and Cox, 2001). Found in the liver and muscles, muscle glycogen is converted into glucose by muscle cells, and liver glycogen converts to glucose for use throughout the body including the Central Nervous System.

In the present study the glycogen content was decreased in both age groups (young and old) of nicotine treatment rats (young by -26.93%; old by -24.04%) when compared to control rats. In red grape extract treatment rats of both (young and old) an increase was observed when compared to the control rats (young by 10.85%; old by 11.89%). In the combination treatment (Nt+RGEt), slightly increased (young by 3.89%; old by 2.74%) was observed when compared to control rats of both age groups (Table.2; Fig 2). From the present investigation it was observed that the lung tissue glycogen levels were decreased in the nicotine treatment (Nt) rats in both age groups. Several authors have been reported decreased glycogen content in afferent tissues with reference to different toxic conditions. Vijayakumar Reddy, (1990) reported decreased glycogen levels in the kidney, liver and muscle, under guanidine toxicity. Hariprasad, (1996) observed decreased Glycogen content in fish with ammonium toxicity. Decrement in tissue glycogen levels has been reported during ammonia stress (Santhi, 1991; Nadamuni Cherry, 1992; Obula Reddy, 1994). The decreased glycogen content in the lung tissue with nicotine treatment rats observed in the present study indicates its greater metabolic utilization possibly to meet higher energy demands to mitigate nicotine toxicity (or) decreased rate of its synthesis. This could be accomplished either through glycolysis (or) the alternative pathway namely the Hexose Monophosphate Pathway (HMP).

In our present findings, it is observed that the glycogen content was increased in RGEt rats in the lung tissue of both age groups when compared to control rats. Red grape extract literature is not available regarding glycogen in this matter. However other evidence indicates that, Shibib *et al*., (1993) reported *Momordica charantia* (Bitter Milon, Family of Cucurbitaceae) fruit juice increase the hepatic glycogen synthesis and decreases the hepatic gluconeogenesis. So that in our studies glycogen content was increased may be due to upregulation of glycogen metabolism by the RGEt rats. From the present investigation it was observed that the lung glycogen levels significantly decreased due to aging (Table; 2; Fig.2). The decrease in the glycogen content with advancement of age may be due to augmented glycogen degradation, through glycolysis or due to decreased in the synthesis of glycogen during aging, Takahashi *et al.,* (1970). The decreased mitochondrial oxidation revealed by decreased activity of ICDH, SDH and MDH clearly indicates the prevalence of hypoxic conditions in the tissues, which normally increases glycogen utilization. In the present investigation elevated glycogen content levels were observed in the lung of both age groups in the combination treatment (Nt+RGEt), suggesting RGEt may beneficial for the nicotine subject to improve the glycogen content under induced nicotine conditions.

Amino acids are the building blocks of proteins, have a pivotal role to play in cellular metabolism. The diverse physical, chemical and biological properties of the proteins are dependent on the nature and arrangement of their constituent's namely free amino acids. A hydrolysis of dietary proteins as well as the breakdown of endogenous proteins result in the of a large amino acid pool in the body conversely amino acids are e precursors for the synthesis of various cellular proteins.

In the present study in total free amino acids content was decreased in both (young and old) nicotine treatment rats (young by –60.39%; old by –41.84%) when compared to control rats. In red grape extract treatment rats of both (young and old) an increase was observed when compared to the control rats (young by 17.17%; old by 23.26%). In the combination treatment (Nt+RGEt) slightly increase (Young by 6.55%; old by 8.03%) was observed when compared to control rats of both age groups (Table. 3; Fig. 3). In the present investigation more amount of free amino acids (FAA) were found in the lung tissue of young age group compared to old age group. Obled and Arnal (1991) suggested that, with advancement of age protein synthesis was decreased and FAA concentration was increased. In general, we can conclude that the age by including tissue proteolysis, elevated free amino acids with a decline in protein synthesis in rats. In the present study total free amino acids content was decreased due to nicotine treatment in both age groups. This decrease may be due to the effect of nicotine products on the FAA content in the lung tissue. However, contradictory reports are also available regarding the influence of nicotine on total free aminoacid pool. Besides these, the enhanced level of FAA may be due to ammonia intoxication (Krishna Mohan Reddy, 1986).

The total FAA content was increased in the RGEt rats, in the both the age groups of lung tissue. Amino acids are added to the pool through the synthesis of non-essential amino acids and precursors within the tissue and through release of amino acids from the breakdown of dietary and cellular proteins in the tissue. The increased amino acid content in the RGEt lung tissue may be due to augmented activity of acidic, alkaline and neutral proteases. This elevation in amino acid level may also be attributed to the enhanced proteolysis as well as decreased amino acid utilization for protein synthesis (Bylund-Fellenius *et al.,* 1984). The low levels of FAA in the lung tissue due to nicotine treatment may also be due to high utilization of these to carbohydrate sources via gluconeogenesis pathway to meet the energy demand under the influence of nicotine intoxication. In the present study we observed an elevation of FAA pool in the lung tissue due to combination (Nt+RGEt), suggests that RGEt enhances the supply of FAA content to counter the nicotine toxicity.

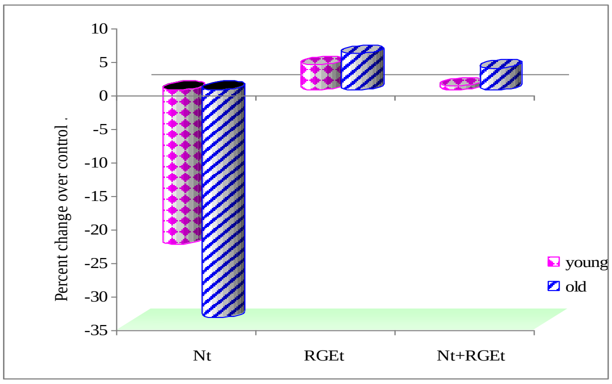

Fig.1: Per cent change over respective control in Total carbohydrates content in lung tissue, of young and old male albino rats in response to Nicotine treatment (Nt), Red Grape Extract treatment (RGEt) and interaction of the both (Nt+RGEt).

Table-1: Changes in Total Carbohydrates content due to Nicotine treatment (Nt), Red Grape Extract treatment (RGEt) and interaction of the both (Nt+RGEt) for a period of 2 months over the control in Lung tissue of male albino rats of young (3 months) and old (18 months) age groups. Values are expressed in mg/gram wet weight of the tissue.

Name of the tissue	Young				Old			
	Control	Nt	RGEt	Nt+ RGEt	Control	Nt	RGEt	Nt+ RGEt
Lung	43.91 ±2.39	33.81** ±1.34 (-23.00)	45.53** ±1.49 (+3.68)	43.25@ ±0.77 (+0.50)	48.25 ±0.85	31.86** ±0.66 (-33.96)	50.83** ±1.02 (+5.34)	49.79@ ±0.88 (+3.19)

All the values are ± SD of six individual observations.
Values in parentheses denote per cent change over respective control.
*Values are significant at $P < 0.05$
**Values are significant at $P < 0.01$
@Values are non significant.

Table-2: Changes in Glycogen content due to Nicotine treatment (Nt), Red Grape Extract treatment (RGEt) and interaction of the both (Nt+RGEt) for a period of 2 months over the control in Lung tissue of male albino rats of young (3 months) and old (18 months) age groups. Values are expressed in mg/gram wet weight of the tissue.

Name of the tissue	Young				Old			
	Control	Nt	RGEt	Nt+RGEt	Control	Nt	RGEt	Nt+RGEt
Lung	56.02 ±0.79	40.93* ±0.68 (-26.93)	62.10** ±0.78 (+10.85)	58.20@ ±0.68 (+3.89)	51.44 ±0.96	37.92** ±2.38 (-24.04)	57.56** ±0.69 (+11.89)	53.48@ ±1.95 (+2.74)

All the values are ± SD of six individual observations.
Values in parentheses denote per cent change over respective control.
*Values are significant at $P < 0.05$
**Values are significant at $P < 0.01$
@Values are non significant.

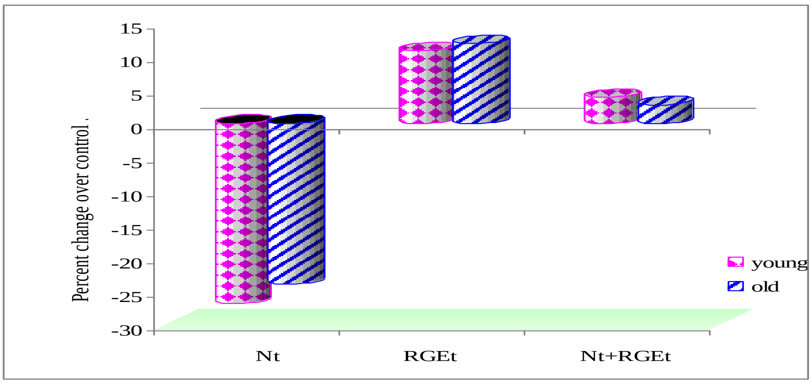

Fig. 2: Per cent change over respective control in Glycogen content in lung tissue, of young and old male albino rats in response to Nicotine treatment (Nt), Red Grape Extract treatment (RGEt) and interaction of the both (Nt+RGEt).

Table-3: Changes in Total free amino acids activity due to Nicotine treatment (Nt), Red Grape Extract treatment (RGEt) and interaction of the both (Nt+RGEt) for a period of 2 months over the control in Lung tissue of male albino rats of young (3 months) and old (18 months) age groups. Values are expressed in mg/gram wet weight of the tissue.

Name of the tissue	Young				Old			
	Control	Nt	RGEt	Nt+RGEt	Control	Nt	RGEt	Nt+RGEt
Lung	28.53 ±0.73	11.30* ±0.66 (-60.39)	33.43** ±0.79 (+17.17)	30.40@ ±1.24 (+6.55)	21.53 ±0.69	12.52** ±0.69 (-41.84)	26.54** ±0.84 (+23.26)	23.26@ ±0.77 (+8.03)

All the values are ± SD of six individual observations.
Values in parentheses denote per cent change over respective control.
*Values are significant at $P < 0.05$
**Values are significant at $P < 0.01$
@Values are non significant

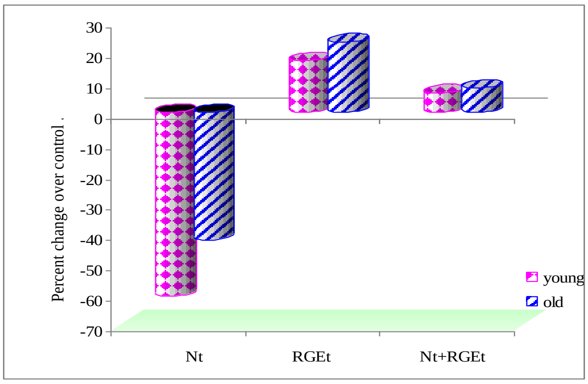

Fig. 3: Per cent change over respective control in Total free amino acid content in lung tissue, of young and old male albino rats in response to Nicotine treatment (Nt), Red Grape Extract treatment (RGEt) and interaction of the both (Nt+RGEt).

CONCLUSION

This investigation draw a conclusion stating that this much 2months red grape extract treatment with the selected mgs (25 mg/kg body weight) that was adapted may be beneficial in countering the age associated changes and nicotine induced alterations in carbohydrate metabolic profiles in the lung tissue.

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