

Significance of red grape extract on oxidative enzymes in the brain of male albino rat with reference to aging

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ABSTRACT

Nicotine has been reported to induce oxidative stress by producing the Reactive Oxygen Species (ROS). In vitro studies showed that Red grape extract has significant antioxidant activity and can inhibit oxidation. Pathogen free, Wistar strain male albino rats were used in the present study, rats were divided into 4 groups of six rats 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 50 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 brain such as the activities of the levels of, Succinate dehydrogenase (SDH), Malate dehydrogenase (MDH), and Isocitrate Dehydrogenase (ICDH) are decreased in nicotine treated rats in the Brain and enhance was observed in the combination (Nt+RGEt), Lactate dehydrogenase (LDH), are increase in nicotine treated rats in the Brain and decreased was observed in the combination (Nt+RGEt), but at 50 mg/kg body weight of Red grape extract found to be more effective. This results stating that Red Grape Extract treated rats are beneficial, especially for the nicotine subjects to improve the brain status. Hence the present study was carried out to evaluate the interaction of nicotine on oxidative enzymes in the brain of male albino rat.

Key words: Nicotine, Red Grape extract, SDH, MDH, ICDH and LDH, Brain and Male albino rat.

INTRODUCTION

The grape has been well recognized worldwide for over 2,000 years as one among the edible sweet fruits and recognized for its wide spectrum of biological properties. Consumption of grape flavonoids has been shown to confer

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antioxidant protection, reduce thrombus formation and lead to the concentration of inflammatory biomarkers (Castilla et al., 2006; O'Byrne et al., 2002). This effect may be considered to be beneficial for the prevention of cardiovascular disease (Castilla et al., 2006). In vitro studies showed that grape juice has significant antioxidant activity and can inhibit oxidation of low density lipoprotein (LDL) (Castilla et al., 2006; O'Byrne et al., 2002). Naissides et al., 2006 said that Oxidative stress is a hallmark of various health problems. Resveratrol is a natural phytoalexin abundantly found in grapes and red wine, which has potent antioxidant property.

Red grape Flavonoids and other plant phenolics have been reported to have multiple

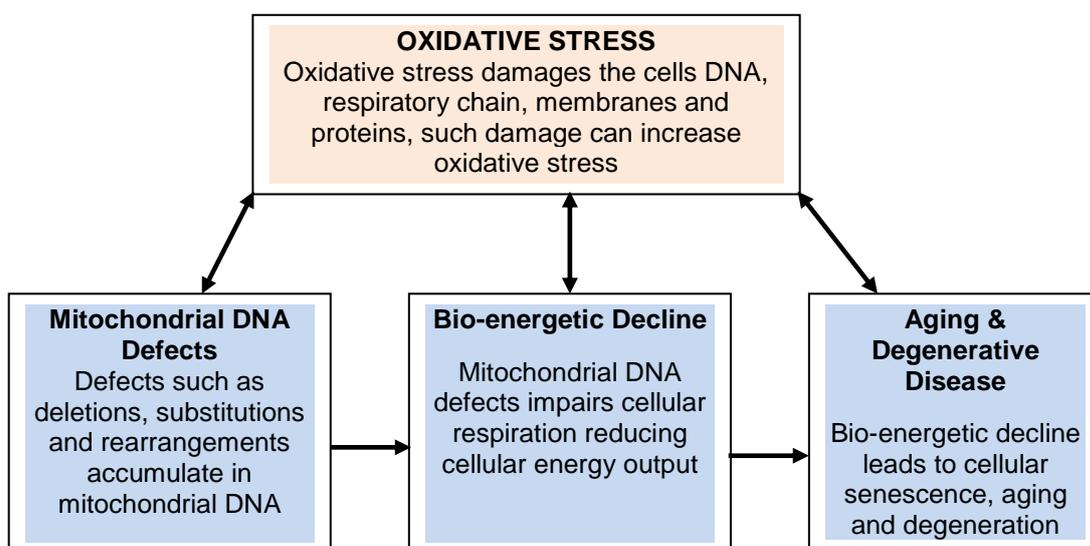
biological effects including antioxidant activity (Frankel *et al.*, 1993), anti-inflammatory action (Moroney *et al.*, 1988), inhibition of platelet aggregation (Kanner *et al.*, 1994) and antimicrobial activity (Renaud and Longenil, 1992). The phenolic compounds mainly include anthocyanins, flavanols, stilbenes (resveratrol) and phenolic acids (Dopico-Garcia *et al.*, 2008; Spacil *et al.*, 2008). Anthocyanins are pigments, and mainly exist in grape skins. The reported evidences of beneficial health effects of phenolic compounds include inhibiting some degenerative diseases, such as cardiovascular diseases (Shanmuganayagam *et al.*, 2007; Tsanga, *et al.*, 2005), and certain types of cancers (God *et al.*, 2007; Jung *et al.*, 2006), reducing plasma oxidation stress and slowing aging (Meyer *et al.*, 1997; Sato *et al.*, 1996). Phenolic compounds are also regarded as preservatives against microbes and oxidation for food (Rodriguez-Vaquero *et al.*, 2007; Rhodes *et al.*, 2006). Flavonoids and other polyphenols found in grapes have the capacity to scavenge Reactive oxygen species (Rice-Evans *et al.*, 1996).

Nicotine is distilled from burning tobacco and carried proximally on tar droplets (also called particulate matter), which are inhaled. Nicotine absorption can occur through the oral cavity, skin, lung, urinary bladder, and gastrointestinal

tract (Yildiz, 2004). Absorption of nicotine across biological membranes depends on pH (Yildiz, 2004). The respiratory absorption of nicotine is 60 % to 80%. (Health Council of the Netherlands, 2004). Nicotine induced 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). In most mammalian species, nicotine is rapidly and extensively metabolized, primarily in the liver (Kyerematen and Vesell, 1991). The major metabolic pathways of nicotine in mammals are C-oxidation and N-oxidation, *i.e.* cotinine and nicotine-1'-N-oxide formation, respectively. Cotinine formation from nicotine is a two-step reaction in mammals. 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).

Reactive oxygen species (ROS) include superoxide and hydroxyl radicals and other activated forms of oxygen such as hydrogen peroxide and singlet oxygen. Free radical damage by various endogenous reactive oxygen species (Finkel and Holbrook, 2000; Harman, 2001). This role of reactive oxygen species in aging (Figure-1) is thought to explain the observation that animals with higher metabolic

Figure-1. The series of boxes on the bottom shows mitochondrial decline can speed up aging and degeneration, as proposed by Linnane.



SOURCE: Linnane, A.W. *et al.*, (1998).

rates have shorter lifespan, the so-called “rate of living” hypothesis (Finkel and Holbrook, 2000).

Nicotine binds to brain tissues with high affinity, and the receptor binding capacity is increased in smokers compared with nonsmokers (Perry *et al.*, 1999). The increase in the binding is caused by a higher number of nicotinic cholinergic receptors in the brain of the smokers. The brain is the most complex organ in a vertebrate's body. In a typical human the cerebral cortex (the largest part) is estimated to contain 15–33 billion neurons. Living brain tissue is pinkish on the outside and mostly white on the inside, with subtle variations in color. Vertebrate brains are surrounded by a system of connective tissue membranes called ‘meninges’ that separate the skull from the brain. The brains of vertebrates are made of very soft tissue. (Kandel *et al.*, 2000).

MATERIALS AND METHODS

Animals

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 male albino rats (Jang *et al.*, 2001) were used in the present study. The usage of animals was approved by the Institutional Animal Ethics Committee (Regd. No. 438/01/a/CPCSEA/dt.17-2-2001) in its resolution number 9/IAEC/SVU/Zool/dt.4-3-2002. 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*.

Procurement of chemicals:

All the chemicals used in the present study were Analytical grade (AR) and obtained from the following scientific companies: Sigma (St. Louis, MO, USA), Fisher (Pittsburg, PA, USA), Merck (Mumbai, India), Ranbaxy (New Delhi, India), Qualigens (Mumbai, India).

Dosage of nicotine:

The dose administration of nicotine was followed as per the protocol given by (Shoab and Stolerman, 1999; Helen *et al.*, 2003) 0.6 mg

/ kg body weight (0.5ml) was chosen as the dose, for this study.

Selection and mode of nicotine treatment:

Nicotine was first distilled from tobacco sap in 1809. Nineteen years later, the main base of tobacco was isolated and separated in pure form from fermented as well as non-fermented tobacco by Posselt and Reimann (Pailer, 1964). They called it nicotine and characterized it as a water-clear liquid, boiling under atmospheric pressure at 246°C, miscible with water, alcohol and ether. Historically nicotine had been recommended for treatment of numerous symptoms.

Physical and chemical properties of nicotine:

Nicotine Scientific name :	<i>Nicotiana tobacco</i>
Nicotine Family :	<i>Solanaceae</i>
Chemical formula:	C ₁₀ H ₁₄ N ₂
Molecular Weight:	162.3
IUPAC Name:	3-[2-(N-methylpyrrolidinyl)]pyridine
Appearance :	Oily, colourless hygroscopic liquid
Characteristic odour :	Turns brown on exposure to air
Boiling point (decomposes) :	246 °C
Density :	1.01 g cm ⁻³
Solubility in water:	miscible

Red Grape Collection and Extraction

Red Grapes, as large clusters with red berries, were brought from local surroundings in Bangalore and identified as *Vitis vinifera* L. (Family *Vitaceae*). The grape were crushed (whole fruit) for juice and dried in shade, powdered and extract by maceration with 70% alcoholic for 72 hours in ambient temperature. The Red Grape extract was filtered and then solvent evaporated to dryness under reduced pressure in a rotary evaporator. The residual Red Grape extract was used for this study.

Treatment schedule:

Age matched rats divided into 4 groups of six in each group and treated as follows:

- Group: i) Control rats (Rats received 0.9% saline). Group:ii) Nicotine treatment (Nt) (Rats were received the nicotine at a dose of 0.6

mg/kg body weight by subcutaneous injection for a period of 2 months).

- Group: iii) Red Grape Extract treatment (RGEt) (Rats were received red grape extract 50mg/kg body weight via orogastric tube for a period of 2 months).
- Group: iv) Nicotine + Red grape extract (Nt+RGEt) (Rats were received the nicotine at a dose of 0.6 mg/kg body weight by subcutaneous injection and red grape extract 50mg/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 brain was isolated at -4°C , washed with ice-cold saline, immediately immersed in liquid nitrogen and stored at -80°C , 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 Investigation:

In the present study Succinate dehydrogenase (SDH), Malate dehydrogenase (MDH), Iso-citrate Dehydrogenase (ICDH), and Lactate dehydrogenase (LDH), were analyzed.

Lactate Dehydrogenase activity was determined by the method described by Nachlas *et al.*, (1960) as suggested by Prameelamma and Swami (1975). The enzyme LDH activity was expressed in μ moles of formazan formed / mg protein/hour. Iso-citrate dehydrogenase (ICDH) was assayed by the method of

Korenberg and Pricer (1951) as modified by Mastanaiah *et al.*, (1978). The enzyme ICDH activity was expressed as μ moles of formazan formed/mg protein/hour. The specific activity of SDH was assayed by the method of Nachlas *et al.*, (1960) as suggested by Prameelamma and Swami (1975) with slight modifications. The activity was expressed in μ moles of formazan formed / mg protein / hour. The specific activity of MDH was measured by the method of Nachlas *et al.*, (1960) as suggested by Prameelamma and Swami (1975) with slight modifications, The activity was expressed in μ moles of formazan formed / mg protein / hour.

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

Lactate Dehydrogenase (LDH):

In the present study the Lactate dehydrogenase activity was increased in both (young and old) nicotine treatment rats (young by 6.71%; old by 10.77%) when compared to control rats. In red grape extract treatment rats of both (young and old) an increase (young by 2.31 %; old by 5.65 %) was observed when compared to control rats. In the combination treatment (Nt+RGEt) slightly increased was observed when compared to control rats of both age groups (Table.1).

Table –1: Changes in Lactate Dehydrogenase (LDH) 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 Brain of male albino rats of young (3 months) and old (18 months) age groups. Values are expressed as μ moles of formazan formed/mg protein/hour.

Name of the tissue	Young				Old			
	Control	Nt	RGEt	Nt+RGEt	Control	Nt	RGEt	Nt+RGEt
Brain	45.44	48.49**	46.49**	47.59 [@]	38.23	42.35**	39.25**	41.25 [@]
	± 6.32	± 5.96 (+6.71)	± 6.75 (+2.31)	± 7.97 (+4.73)	± 9.36	± 5.84 (+10.77)	± 6.05 (+2.66)	± 5.25 (+7.89)

All the values are \pm SD of six individual observations.

Values in parentheses denote per cent change over respective control.

** Values are significant at $P < 0.01$

[@] Values are non-significant.

Table–2. Changes in Iso-citrate dehydrogenase (ICDH) 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 Brain of male albino rats of young (3 months) and old (18 months) age groups. Values are expressed as m moles of formazan formed/mg protein/hour.

Name of the tissue	Young				Old			
	Control	Nt	RGEt	Nt+RGEt	Control	Nt	RGEt	Nt+RGEt
Brain	96.24	63.19**	115.58**	108.12@	85.45	65.82**	90.27**	87.80@
	±7.32	±4.38 (-34.34)	±11.84 (+20.09)	±9.60 (+12.34)	±4.99	±7.53 (-22.97)	±6.69 (+5.64)	±7.53 (+2.75)

All the values are \pm SD of six individual observations.

Values in parentheses denote per cent change over respective control.

** Values are significant at $P < 0.01$

@ Values are non-significant.

The rats which, received nicotine showed an elevation of LDH activity in the brain tissue in both the age groups. Moreover, the high per cent elevation of LDH was noticed in old (by 10.77%) group than the young group (6.71%) compared to their respective control rats. Several authors have reported increased LDH activity in afferent tissues with reference to different toxic conditions. LDH is a cytosolic enzyme, which allows the assessment of the process of anaerobic energy production by the cell. The LDH was increased due the nicotine treatment chronic as well as acute in brain tissue (Turegano *et al.*, 2001). This enzyme is a marker of metabolic activity of renal glomeruli. A dose-dependent increase in LDH activity, evident after 24 weeks of cadmium (Cd) exposure, in the main tubules and glomeruli reflects intensification of anaerobic respiration (Malgorzata *et al.*, 2004). Cunningham and Ivester, (1999) reported that significant ethanol-related increase in lactate dehydrogenase activity released from both periportal and perivenous cell that occur under toxic conditions or at low oxygen tensions. The release of LDH was greatest in perivenous-ethanol hepatocyte, but was significantly different from control hepatocytes in both cell types. Strubelt *et al.*, (1999). According to Yildiz D *et al.*, (1999) LDH activity was increased due to nicotine induced oxidative stress. In our present investigations, the increased levels of LDH activity in nicotine treatments. This is due to the increased generation of ROS by nicotine that leads to cell

damage and also indicated the low capacity to combat against ROS.

The brain tissue LDH activity was increased with RGEt rats in both age groups when compared to control rats. This reports suggesting enhanced oxidative metabolism in RGEt rats to meet the increased energy demands of the animal. An increase in NAD dependent LDH activity in the brain tissue of rat subjected to RGEt, indicate the possible shift in the metabolic profile from the anaerobiosis to aerobiosis i.e., the NAD-LDH activity helps in the efficient conversion of lactate to pyruvate and its subsequent utilization in TCA cycle oxidative reactions. The lactate taken up by the tissue may be oxidized to carbon dioxide and water or used for glycogenesis. In both cases pyruvate is the first product (Rasmussen *et al.*, 2002). Due to increased lactate levels in the brain tissue, the LDH activity may also increase to convert the high amount of lactate to pyruvate during red grape extract treatment (RGEt).

However, the direction of LDH is determined by the lactate / pyruvate ratio multiplied by the NAD / NADH ratio' Lactate oxidation only occurs if this mass action ratio is larger than the equilibrium constant (Rasmussen *et al.*, 2002). These evidences support the age related increased in LDH activity in old age rat of brain tissue, however in the combination treatment (Nt+RGEt) the LDH activity was increased in both age groups.

Isocitrate Dehydrogenase (ICDH):

In the present study in iso-citrate dehydrogenase content was decreased in both (young and old) nicotine treatment rats (young by -34.34 %; old by (-22.97%) when compared to control rats. In red grape extract treatment rats of both (young and old) an increased was observed when compared to the control rats (young by 20.09 %; old by 5.64 %). In the combination treatment (Nt+RGEt) slightly increased was observed when compared to control rats of both age groups (Table.2).

In the present study a decrease of ICDH activity was observed in brain tissue of both the age groups of nicotine treatment rats. The decrease in specific activity of NAD/NADP-ICDH as a consequence of induced nicotine toxicity suggests reduced conversion of isocitrate to α -Ketoglutarate. Similar changes in ICDH activity was reported in different animals treated with various toxic compounds (Joseph and Rao, 1990; Reddy and Rao, 1991). The changes in NADP-ICDH could be attributed to the mitochondrial damage caused by nicotine treatment. Interaction of enzyme with NADPH may result in an unfavorable conformation of the enzyme molecule (Plaut, 1963). The brain NADP-ICDH was decreased, suggesting reduced mitochondrial oxidation of isocitrate in brain with advancement of age. This could be credited to diminished supply of keto acids into citric acid cycle (Thalwar *et al.*, 1989). Sanadhi, (1967) reported that the transfer of substrate by

the mitochondrial membrane is altered in old cells because of rupture of the membrane. Age-dependent damage in individual process has been reported for several enzymes including mitochondrial oxidoreductases (Thalwar *et al.*, 1989).

In the present investigation we observed a slight/marginal increase in ICDH activity when the nicotine treatment rats were supplemented by the (combination treatment Nt+RGET). This restoration of ICDH activity reveals the normal operation of TCA cycle for high energy production to withstand the toxic conditions of nicotine metabolic profiles. This observation which is a beneficial to the organism to streamline the deranged metabolic machinery either due to aging or nicotine toxicity.

Succinate Dehydrogenase (SDH):

In the present study the succinate dehydrogenase activity was decreased in both (young and old) nicotine treatment rats (young by -41.39 %; old by -24.49%) when compared to control rats. In red grape extract treatment rats of both (young and old) an increased was observed when compared to the control rats (young by 24.25 %; old by 16.55 %). In the combination treatment (Nt+RGEt) slightly increased was observed when compared to control rats of both age groups (Table.3).

The decrease in SDH activity due to the nicotine stress condition indicates reduction in

Table–3. Changes in Succinate dehydrogenase (SDH) 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 Brain of male albino rats of young (3 months) and old (18 months) age groups. Values are expressed as m moles of formazan formed/mg protein/hour.

Name of the tissue	Young				Old			
	Control	Nt	RGEt	Nt+RGEt	Control	Nt	RGEt	Nt+RGEt
Brain	94.72	55.51**	117.69**	97.42@	79.12	59.74**	92.22**	82.28@
	±4.59	±11.45 (-41.39)	±15.69 (+24.25)	±8.644 (+2.85)	±7.98	±6.04 (-24.49)	±5.35 (+16.55)	±5.95 (+3.99)

All the values are \pm SD of six individual observations.

Values in parentheses denote per cent change over respective control.

** Values are significant at $P < 0.01$

@ Values are non-significant.

the conversion of succinate to fumarate resulting in decreased oxidative metabolism. Similar inhibition of SDH activity was reported in animals under induced different toxic conditions (Hamilton and Gould, 1987; Veerababu, 1988; Gupta *et al.*, 1991). Chennaiah *et al.*, (2011) reported the decreased SDH activity was observed in all skeletal muscle fibres of rats treated with nicotine, indicating depressed oxidative metabolism in mitochondria. Since the activity of SDH is reduced, it is evident that this might affect the conversion of malate to oxaloacetate by MDH because of low succinate oxidation. A decrease in oxygen consumption in stress condition also leads to inhibition of mitochondrial oxido-reductases (Moorthy *et al.*, 1985). The reduced availability of oxidized form of flavoproteins needed for succinate oxidation results in decreased activity of SDH (Swami *et al.*, 1983).

The SDH activity was increased in the brain tissue of both the age groups supplemented with RGEt when compared to the control rats. The increase in maximal and specific activity of SDH by RGEt suggests the increased mitochondrial oxidative potential and energy synthesis utilizing carbohydrates and fats as substrates. In the present study an increase was observed in the RGEt rats of both the age groups. The increase in specific activity of SDH in old age rats with response to RGEt suggests the increased mitochondrial oxidative potential and energy synthesis utilizing carbohydrates and fats as substrates function of mitochondria is energy production, isolated mitochondria generate

reactive oxygen species during oxidative phosphorylation.

There is surprisingly little direct evidence for the generation of reactive species by mitochondria in intact cells of tissue (Leeuwenburgh and Heinecke, 2001). In the combination treatment with (Nt+RGEt) upregulation was observed in the brain tissue of both age groups. Thus differential response of SDH activity was observed in the brain tissue of both age groups in the present study.

Malate Dehydrogenase- (MDH):

In the present study the malate dehydrogenase activity was decreased in both (young and old) nicotine treatment rats (young by -47.65 %; old by -27.39%) when compared to control rats. In red grape extract treatment rats of both (young and old) an increased was observed when compared to the control rats (young by 31.28 %; old by 25.47 %). In the combination treatment (Nt+RGEt) slightly increased was observed when compared to control rats of both age groups (Table.4).

A significant decrease in the specific activity of NADP-ICDH (Table.4) and as a consequence of nicotine-treatment observed in the present study indicates reduced formation of malate. The decrease in activity levels of dehydrogenases is consistent with the decreased CO₂ formation (Cederbaum *et al.*, 1976). An increase in proteolytic activity during nicotine intoxication may also be responsible for the decreased MDH activity. A similar study,

Table–4. Changes in Malate dehydrogenase (MDH) 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 Brain of male albino rats of young (3 months) and old (18 months) age groups. Values are expressed as m moles of formazan formed/mg protein/hour.

Name of the tissue	Young				Old			
	Control	Nt	RGEt	Nt+RGEt	Control	Nt	RGEt	Nt+RGEt
Brain	43.25 ±9.85	22.64** ±8.99 (-47.65)	56.78** ±8.38 (+31.28)	49.79@ ±5.10 (+15.12)	35.95 ±5.86	26.10** ±11.67 (-27.39)	45.11** ±8.79 (+25.47)	38.13@ ±8.24 (+6.06)

All the values are ± SD of six individual observations.

Values in parentheses denote per cent change over respective control.

** Values are significant at $P < 0.01$

@ Values are non-significant.

Chennaiah *et al.*, (2011) reported a decrease in specific activity of MDH was observed in the muscle fibers of rats treated with nicotine, suggesting decreased utilization of malate. The decreased MDH activity could be attributed to low availability of substrate, lesser conversion of succinate-fumarate-malate, and the changes in the structural integrity of mitochondria. Similar inhibition of MDH activity was reported in animals under different toxic conditions (Veerababu, 1988; Tripathi and Shukla, 1990; Reddy and Yellamma, 1991). The decrease in specific activity of MDH in brain tissue of both age groups of rats as a consequence nicotine treatment suggests decreased utilization of malate. The reduced levels of TCA cycle intermediates may also be due to the decrease in MDH activity during nicotine-treatment.

Mitochondrial dysfunction and accumulation of protein damage have been proposed to contribute to aging process (Bakala *et al.*, 2003). It has been recently demonstrated that impairment in mitochondrial respiration and oxidative phosphorylation elicits an increase in oxidative stress (Sateesh Pujari and Estari Mamidala, 2015; Yan and Sohal, 1998 and Sivasankar et al, 2014). In recent years, much data has been accumulated to suggest that mitochondria act like a timer that ticks all the way through the aging process (Wei and Lee, 2002). From the present study we report that the combination treatment (Nt+RGET) exhibits a beneficial recovery of MDH activity in both the age groups of brain tissue. This suggests that red grape extract treatment is very much useful for the nicotine subjects to upregulate the decreased oxidative metabolism.

CONCLUSION

This investigation draw a conclusion stating that this much of red grape juice extracts to the old age as well as young age male subjects may be beneficial, especially for the nicotine subjects to improve the health status and life span. The activities were inhibited in Brain tissues of rats treated with Nicotine. In conclusion, the present study shows that red grape juice extracts treatment mitigates nicotine intoxication-induced oxidative damage, which could be due its antioxidant nature that combines free radical scavenging and metal chelating properties.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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