Original Paper

Effect of Red Grape Extract on Nicotine-induced Oxidative Stress in Skeletal Muscle Fibres of Male Albino Rat

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ABSTRACT

In this study we have assessed the protective effect of red grape extract against nicotine-induced oxidative damage. Pathogen free, Wistar strain, male albino rats were used in the study. Age-matched rats were divided into 4 groups of six in each group and treated as follows: i) Normal Control (NC) (*Control rats that received 0.9% saline*) ii) Nicotine treated (Nt) (*0.6 mg/kg body weight by subcutaneous injection for a period of 2 months*) iii) Nicotine treated + Red grape extract treated (Nt+RGEt-25) (*nicotine at 0.6 mg/kg by subcutaneous injection and red grape extract at 25 mg/kg body via orogastric tube for a period of 2 months*) iv) Nicotine treated + Red grape extract treated (Nt+RGEt-50) (*nicotine at 0.6 mg/kg by subcutaneous injection for a period of 2 months, red grape extract at 50 mg/kg via orogastric tube for a period of 2 months*).

The animals were sacrificed after 24 hrs after the last treatment by cervical dislocation and the skeletal muscle fibres such as Gastrocnemius (GN) and Soleus (SOL) were isolated, and the activities of alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LDH) were estimated.

In nicotine treated-rats, the activities of ALP, ALT, AST and LDH were significantly increased in both muscles when compared to control rats. In the combination treatment groups (nicotine + red grape extract) upregulation was observed, red grape at a dose of 50 mg/kg body weight being found to be more effective. The levels of glutathione (GSH), glutathione peroxidase(GPx), superoxide dismutase(SOD) and catalase (CAT) were decreased in nicotine-treated rats in both muscles, and increase was observed in the combination groups (Nt+RGEt), red grape at a dose of 50 mg/kg body weight being found to be more effective.

These results indicate that red grape extracts are beneficial, especially for nicotine-exposed subjects to improve the metabolic efficiency, and thereby improve the health status and life span.

Key Words: Nicotine; Red grape extract; Marker enzymes; Antioxidants; Skeletal muscles

Introduction

Nicotine, the major component of cigarette smoke plays an important role in the development of cancer and lung related complications.[1] Among the most well characterized chemicals found in tobacco and tobacco smoke, are polycyclic aromatic hydrocarbons (PAHs) and the highly addictive alkaloid, nicotine and its metabolites.[2] To further complicate the picture, nicotine is converted, during the production of cigarette and chewing tobacco, into two highly mutagenic nitrosamines, N-nitrosonornicotine (NNN) and 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK), and is metabolized into cotinine. These chemicals derivatives also exhibit a wide spectrum of biological activity as compared to the parent compound.[2] Nicotine has been reported to induce oxidative stress both in vivo and in vitro.[3] The mechanism of generation of free radicals by nicotine is not clear. But oxidative stress

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occurs when there are excess free radicals and/or low antioxidant defense, and result in chemical alteration of biomolecules causing structural and functional modification. Oxygen free radicals (OFR) production has been directly linked to oxidation of cellular macromolecules, which may induce a variety of cellular responses through generation of secondary metabolic reactive species.[4] Previous reports have shown enhanced lipid peroxidation and inadequate antioxidant status by nicotine.

The rapid elimination of nicotine has been attributed to its metabolism, as well as distribution to some tissues. The liver plays a major role in the detoxification and elimination of foreign compounds. The predominant effects of nicotine in the whole intact animal or human consist of an increase in rate blood pressure, increase in plasma free fatty acids and lung injury.[5] Nicotine has been extensively studied in separately designed in vivo and in vitro experimental systems using either nicotine or smokeless tobacco extract. Some of the biological and physiological end points of tobacco consumption have been attributed to its major alkaloid, nicotine.

Grapes (*Vitis vinifera*) are among the world's widely grown fruit crops with an annual production of 58-61 million metric tons.[6] Grape growing plays a major role in the worldwide fruit production, with an international acreage of approximately 7.8 million hectares.[7] In the 1980s, there was a high rate of global production of fresh grapes, but due to a reduction in the production surface area, there was a drop in the beginning of the 1990s. However, soon after, the production rate plummeted, due to an increase in output trends, favourable climatic conditions and increase in the partial geographical redistribution of vineyards during this period. America saw its 2005 production reach a record high with 142.6 Mqx (millions of quintals).[8] Anthocyanins tend to be the main polyphenolics in red grapes, whereas flavan-3-ols (e.g., catchiness) are the more abundant phenolic in white varieties.[10] Total phenolic content, an index of dietary antioxidant strength, is higher in red varieties due almost entirely to anthocyanin density in red grape skin compared to absence of anthocyanins in white grape skin. It is these anthocyanins that are attracting the efforts of scientists to define their properties for human health.[9] Phenolic content of grape skin varies with cultivar, soil composition, climate, geographic origin, and cultivation practices or exposure to diseases such as fungal infections. This study was designed to investigate the effects of red grape extracts on nicotine-induced oxidative stress in the skeletal muscle fibres of male albino rat.

Materials and Methods

Animals: Male pathogen-free Wistar albino rats were obtained from the Department of Zoology, Animal House, S.V.U PG Centre, Kavali, Andhra Pradesh. The animals were housed six to a polypropylene cage and provided with food and water ad libitum. The animals were maintained under standard conditions of temperature and humidity with an alternating 12hr light/dark cycle. Standard pellet diet (Agro Corporation Pvt. Ltd., Bangalore, India) was administered, and the animals were maintained in accordance with the guidelines of the National Institute of Nutrition and Indian Council of Medical Research, Hyderabad, India.

Chemicals: Nicotine and other fine chemicals were obtained from Sigma Chemical Company, St. Louis, USA. All other chemicals and reagents used were of analytical grade.

Red grape extraction: Grapes, as large clusters with red berries, were bought from a local supermarket in Chennai and identified as *Vitis vinifera*. Grape seeds and skin were removed from the grapes, the grape pulp was crushed for juice and dried in shade, powdered and extracted by maceration with 70% (v/v) alcoholic for 72 hrs in ambient temperature. The extract was filtered and the solvent evaporated to dryness under reduced pressure in a rotary evaporator. The residual extract was used for the study.

Experimental design: Age-matched rats were 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): These rats received nicotine at a dose of 0.6 mg/kg body weight by subcutaneous injection for a period of 2 months.

Group III – Nicotine treated + Red Grape extract treated (Nt+RGEt): These rats received nicotine at a dose of 0.6 mg/kg body weight by subcutaneous injection and red grape extract 25 mg/kg body weight via orogastric tube for a period of 2 months.

Group IV – Nicotine treated + Red Grape extract treated (Nt+RGEt): These rats received nicotine at a dose of 0.6 mg/kg body weight by subcutaneous injection and red grape extract 50 mg/kg body weight via orogastric tube for a period of 2 months.

The animals were sacrificed 24 hrs after the last treatment session by cervical dislocation, and skeletal muscle fibres (from gastrocnemius and soleus) were isolated at 4^0C, washed with ice-cold saline, immediately immersed in liquid nitrogen and stored at -80^0C 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 investigations: The activity of alkaline phosphatase (ALP) was estimated by the method of King and Armstrong,[11] aspartate transaminase (AST) and alanine transaminase (ALT) by the method of Rertman and Frankel,[12] lactate dehydrogenase (LDH) by the method of Young,[13] total reduced glutathione (GSH) by the method of Ellman,[14] glutathione peroxidase (GPx) by the method of Rotruck et al,[15] superoxide dismutase (SOD) by the method of Kakkar et al,[16] and catalase (CAT) by the method of Sinha.[17]

Statistical analysis: Statistical analysis was carried out using INSTAT software. The data was analyzed for significance, and the results were presented with p-values.

Results

In nicotine-treated rats, the activities of ALP, ALT, AST and LDH were significantly increased when compared to the control. Administration of red grape extract to nicotine-treated rats at different doses significantly decreased the activities of these enzymes when compared with the nicotine-treated animals, and red grape extract at the dose of 50 mg/kg body weight was found to be more effective (**Table 1**).

Table 2 shows that the levels of GSH and GPx were decreased significantly in the nicotine-treated group in both muscles. Significant protection was seen in the red grape supplemented nicotine-treated animals, 50 mg/kg body weight being found to be more effective than the other dose.

Both SOD and CAT were significantly decreased in the nicotine-treated muscles. The activities of SOD and CAT were significantly elevated when red grape extract was added to the nicotine-treated animals, and the dose at 50 mg/kg body weight was found to be more effective when compared to 25 mg/kg body weight (**Table 3**).

Discussion

Knowledge of the toxicity of nicotine is important to understand tobacco-induced human diseases, as well as to assess the potential risks associated with the therapeutic use of nicotine as an aid to assist smoking cessation. Many drugs and chemicals have been shown to induce toxic side effects, and adverse or beneficial effects on multiple enzymes and metabolic processes. For example, a diuretic drug, acetazolamide inhibits carbonic anhydrase. Inhibition of specific enzymes due to drug use may exert pathological states in experimental models, for example, chronic nicotine administration inhibits cytochrome P450 (CYP2Abin liver, CYP1A1 in lungs) as well as generates free radicals, and exerts oxidative tissue injury.[18]

Table 1 Activities of Marker Enzymes in GN and SOL (mean ±SD; n= 6)

Name of muscle	Group	ALT	AST	ALP	LDH
Gastrocnemius (GN)	I) NC	10.84 ± 1.64	20.64 ±1.06	30.38 ± 0.85	43.68 ± 1.30
	II) Nt	16.50 ± 1.53*	25.06 ± 2.57*	36.55 ± 0.79*	47.21 ± 1.89*
	III) Nt+RGEt (25 mg)	14.69 ± 1.68**	24.42 ± 1.40*	34.46 ± 0.79*	45.92 ± 1.42**
	IV) Nt +RGEt (50 mg)	12.01 ± 1.41*	23.66 ± 1.40*	31.51 ± 0.72*	44.79 ±1.01*
Soleus (SOL)	I) NC	6.92 ± 1.47	16.78 ± 1.79	26.86 ± 0.63	36.86 ± 0.61
	II) Nt	9.73 ± 1.16*	21.96 ± 1.90*	31.31 ± 0.79*	41.78 ± 1.72*
	III) Nt+RGEt (25 mg)	7.65 ± 1.61*	19.59 ± 1.66**	28.45 ± 0.79*	38.25 ±1.49**
	IV) Nt+RGEt (50 mg)	6.17 ± 1.24*	17.48 ± 1.29*	27.33 ± 1.03**	37.91 ±1.66**

*Values are significant at $p < 0.001$, **Values are significant at $p < 0.01$

Table 2 Levels of GSH and GPx in GN and SOL (mean ±SD; n= 6)

Name of muscle	Group	GSH	GPx
Gastrocnemius (GN)	I) NC	30.56 ± 1.60	20.62 ± 0.95
	II) Nt	22.83 ± 1.07*	16.71 ± 0.62*
	III) Nt+RGEt (25 mg)	25.32 ± 1.50**	18.07 ± 1.53*
	IV) Nt +RGEt (50 mg)	28.92 ± 1.83*	19.10 ± 1.88*
Soleus (SOL)	I) NC	26.13 ± 1.55	12.66 ± 2.06
	II) Nt	18.95 ± 0.90*	8.80 ±1.48*
	III) Nt+RGEt (25 mg)	22.55 ± 0.74*	10.61 ± 2.53**
	IV) Nt+RGEt (50 mg)	24.05 ± 1.37*	11.21 ± 1.96*
*Values are significant at p < 0.001, **Values are significant at p < 0.01			

The generation of oxygen free radicals can be prevented or scavenged by host antioxidant defense mechanism.[19]

The metabolites of nicotine cause a significant increase in DNA strand breakage.[20] Nicotine also causes oxidative damage which is prominent in the lungs, kidney, brain and liver.[21] This leads to increase in the levels of reactive oxygen species and lipid peroxidation.[22] Lipid peroxidation and generation of free radicals are the process associated with the pathogenesis of many diseases. In this study, the marker enzymes, namely ALP, ALT, AST and LDH were elevated (**Table 1**) in both muscles (GN, SOL) of nicotine-treated rats. These marker enzymes are the important index for the diagnosis of lung diseases and it indicates the damage of cells, cellular leakage and loss of functional integrity of cell membrane.[23] Alkaline phosphatase (ALP) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. AST and ALT are enzymes found in the cytoplasm which appear in cell and is elevated during cellular necrosis.[24] LDH is a cytoplasmic enzyme, which is used to detect cell damage or cell death which takes place during an influx of polymorphonuclear neutrophils and activation of alveolar macrophages.[25] The activity of marker enzymes decreased in the red grape extract

Table 3 Activities of SOD and CAT in GN and SOL

Name of muscle	Group	SOD	CAT
Gastrocnemius (GN)	I) NC	10.86 ± 2.12	25.52 ± 1.20
	II) Nt	22.83 ± 1.07*	16.71 ± 0.62*
	III) Nt+RGEt (25 mg)	25.32 ± 1.50**	18.07 ± 1.53**
	IV) Nt +RGEt (50 mg)	28.92 ± 1.83*	19.10 ± 1.88*
Soleus (SOL)	I) NC	26.13 ± 1.55	12.66 ± 2.06
	II) Nt	18.95 ± 0.90*	8.80 ±1.48*
	III) Nt+RGEt (25 mg)	22.55 ± 0.74**	10.61 ± 2.53**
	IV) Nt+RGEt (50 mg)	24.05 ± 1.37 *	11.21 ± 1.96*
*Values are significant at p < 0.001, **Values are significant at p < 0.01			

rats in both muscles; red grape extracts reversed the changes induced by nicotine in rats, and is more in 50 mg/kg than the 25 mg/kg treated group.

Glutathione (GSH) is a major non-enzymatic antioxidant, and the most abundant non-proteine thiol source in the cell. It has several important functions related to free radical metabolism. GSH is a major non-protein thiol in living organisms which plays a central role in coordinating the body's antioxidant defense process.[26] Perturbation of GSH status of a biological system has been reported to lead to serious consequences. Hussain et al[27] reported that chronic administration of nicotine significantly depletes GSH content in liver and testes. In our study, a significant decrease in the GSH levels was demonstrated in both muscle types of albino rat (**Table 2**). GSH depletion is due to the destruction of free radicals.[28] The decreased levels of tissue GSH in nicotine-treated rats may be due to the enhanced utilization during detoxification of nicotine. Decrease in GSH in tissues leads to oxidative tissue damage.[27] The increase in GSH levels in the red grape extract treated rats (because of the antioxidant properties of red grape extract), was found more in 50 mg/kg than the 25 mg/kg group.

GPx, SOD and CAT constitute a mutually supportive team of defense against reactive oxygen species which have been found decreased in nicotine treated rats in this study (**Table 2, Table 3**). SOD is a ubiquitous enzyme with an essential function in protecting aerobic cells against oxidative stress. It is primarily a mitochondrial enzyme, usually found in the plasma membrane. Catalase is a tetrameric haemoprotein that undergoes alternate divalent oxidation and reduction at its active site in the presence of H_2O_2 and catalyzes the dismutation reaction.[29] GPx is a seleno enzyme two thirds of which is present in cytosol and one third in mitochondria.[30] It catalyses the reaction of hydroperoxides with reduced glutathione to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide.[31] Depletion of the activities of SOD, CAT and GPx in the nicotine-treated rats may be due to the increased utilization of these antioxidants to counter lipid peroxidation. Regarding skeletal muscle and nicotine metabolism, availble literature is not clear. Ashakumari and Vijayammal[32] reported a significant fall in SOD activities in nicotine-treated tissues. Chennaiah[33] reported that in case of high nicotine toxicity, SOD was decreased in the muscle of rat. The depletion in SOD activity may be due to free radicals produced as a result of nicotine toxicity (**Table 3**). The activity of SOD increases in the red grape extract treated rats in both muscles; red grape extracts reversed the changes induced by nicotine in rats. It enhanced the antioxidant status in the tissues of nicotine-treated rats.

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