

Augmented oxidative stress in hyperglycemic albino mice exposed to cigarette smoke

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Abstract

Aim: Our experiment aimed to evaluate the effects of sidestream cigarette smoke (CS) exposed to diabetic mice. Oxidative stress was evoked by CS and hyperglycemia in albino mice. **Materials and Methods:** The mice were exposed to CS of two cigarettes for 15 min, twice daily 6 days a week for 16 weeks. After the experimental period, the mice in the different groups were sacrificed by decapitation. Lung tissue was collected in an ice-cold container to carry out biochemical estimation. Oxidative stress was assessed in experimental groups. **Results and Discussion:** The serum insulin levels were recorded a significant depletion in Groups II, III, and IV when compared to that of control animals. The levels of thiobarbituric acid reactive substances and activity levels of glutathione-s-transferase were significantly increased in experimental groups when compared with control groups. The antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase were significantly depleted in the experimental animals when compared to that of normal animal. **Conclusion:** Oxidative stress induced by sidestream CS, hyperglycemia, and both in combination aggrandize lipid peroxidation.

Key words: Cigarette smoke, hyperglycemia, reactive oxygen species

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder is characterized by hyperglycemia due to abnormal insulin secretion and/or insulin receptor or post-receptor event affecting metabolism involving carbohydrates, proteins, and fats in addition to damaging liver, lungs, kidneys, and β cells of the pancreas.^[1] Diabetes is a disease, in which the hallmark feature is elevated blood glucose concentrations due to a loss of insulin-producing pancreatic β -cells (type 1 diabetes) or through loss of insulin responsiveness in its target tissues such as adipose and muscle (type 2 diabetes). Type 1 diabetes usually begins to manifest in childhood and early adulthood, but type 2 diabetes is typically a disease for which increased age is a risk factor.^[2] Smoking and diabetes are two important hazards to the health of many individuals and contribute substantially to the global burden of disease in various ways. Smoking can not only aggravate the diabetes complications such as macro- or micro-vascular disease but has also been shown to deteriorate glucose metabolism in normal subjects and

thereby may provoke the onset of type 2 diabetes.^[3] It is a major public health problem that is associated with high mortality and morbidity,^[4] as prolonged exposure to hyperglycemia is now recognized as a major factor in the pathogenesis of diabetic complications.^[5] Consequently, the kidneys, eyes, cardiovascular system, and respiratory system can be damaged.^[6] Biochemical pathways strictly associated with hyperglycemia (non-enzymatic glycosylation, glucose autoxidation, and polyol pathways) can increase the production of free radicals,^[7] and their abnormally high level to tissue damage and enzyme dysfunctions.^[8]

Oxidative stress was evoked by cigarette smoke (CS) and diabetes and both in combination enhanced lipid peroxidation. Reactive oxygen species (ROS) can attack all types of

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macromolecules including protein, lipid, and DNA;^[9,10] in addition, oxidative stress is involved in membrane disruption, lipid peroxidation, mutagenic, and carcinogenic process (CS).^[11] Hyperglycemia-induce generation of free radicals (OS) contributes to the development and progression of diabetes and other related complications.^[12] Diabetes along with CS exposure enhanced the generation of oxygen free radicals. The free radicals and oxidant in CS are responsible for lipid peroxidation in lung tissue of mice. The massive surface area makes the lung a target organ for oxidative stress due to CS. A decrease in enzymatic and non-enzymatic antioxidants of defense mechanisms might be an indication of a lipid peroxidation.

In this study, the oxidative stress was assessed in the streptozotocin (STZ) evoked diabetic animals exposed to CS. The impact of CS exposure on the diabetic animals by studying biochemical parameters pertaining to oxidative stress and bronchoalveolar lavage fluid was assessed for alveolar macrophage (AMs) apoptosis.

MATERIALS AND METHODS

Experimental Animals

Adult male albino mice of Wistar strain, weighing 25-30 g, were purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai, India. The animals were kept in polypropylene cages (three in each cage) at an ambient temperature of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and 55-65% relative humidity. A 12 ± 1 h light and the dark schedule were maintained in the animal house till the animals were acclimatized to the laboratory conditions and were fed with commercially available mice chow (Hindustan Lever Ltd., Bangalore, India) and had free access to water. The experiments were carried out in accordance with the guidelines provided by the Institutional Animal Ethical Committee.

CS Exposure

The smoke apparatus consists of four major parts including a power supply, a cigarette burn box, a circulation fan, and inhalation chamber. The cigarette burn box has a slide glass door for controlling air supply and handling cigarette. The circulation fan is connected to the cigarette burn box and blows sidestream CS into the inhalation chamber sized 30 cm (length) \times 20 cm (width) \times 25 cm (height). A burning cigarette was introduced through one hole and fixed to a holder, and air with a pressure of 0.4 kg/cm^2 was passed through the other. The 15 min CS exposure time was chosen because it is the time enough to burn the cigarettes completely.^[13]

Experimental Induction of Diabetes

Diabetes was evoked in the overnight fasted animals by a single intraperitoneal injection of freshly prepared solution

of STZ (Sigma, USA) 200 mg/kg body weight in 0.1M cold citrate buffer pH 4.5.^[14] The animals were considered diabetic if the blood glucose values were $>250 \text{ mg/dl}$ on the third day STZ injection.

Experimental Protocol

The mice were randomly divided into four groups of eight mice's each.

Group I: Mice (control) were exposed to ambient air.

Group II: Mice (experiment) were exposed to CS for 16 weeks.

Group III: Diabetic mice were exposed air (Sham) animals.

Group IV: Diabetic animals exposed to CS for 16 weeks.

The animals were exposed to sidestream CS of two cigarettes for 15 min, twice daily 6 days a week for 16 weeks as described earlier.^[13,15]

Preparation of Lung Tissue and Serum Samples

At the end of the experimental period, mice were anesthetized with sodium pentobarbital (35 mg/kg i.p) and then sacrificed by decapitation. The blood was collected from the mice and centrifuged. The serum samples were collected in separate containers for biochemical estimations. Lung tissues were also collected in ice-cold container for various biochemical estimations.

Estimation of Insulin

Plasma insulin was estimated using RIA assay kit for rats supplied by Ljico Research Inc. (Stat Diagnostics, Mumbai, Maharashtra, India).

Estimation of Lipid Peroxidation

Estimation of thiobarbituric acid reactive substances (TBARS)

The level of TBARS in lung tissue was estimated by measuring malondialdehyde and TBARS reactivity with TBA to generate a pink color chromophore, which was read at 535 nm by Niehaus and Samuelson.^[16] The transmissions were measured by calorimeter and expressed in terms of mM/100 g wet tissue.

Estimation of superoxide dismutase (SOD) (EC 1.15.1.1)

SOD was assayed by utilizing the method of Kakkar *et al.*^[17] A single unit of enzyme was expressed as 50% inhibition of nitroblue tetrazolium (NBT) reduction/min/mg protein.

Estimation of catalase (CAT) (EC.1.11.1.6)

CAT was assayed colorimetrically at 620 nm and expressed as Mmoles of H_2O_2 consumed/min/mg protein as described

by Sinha.^[18] The reaction mixture 1.5ml contained 1.0 ml of 0.01M pH 7.0 phosphate buffer, 0.1 ml of tissue homogenate, and 0.4 ml of 2M H₂O₂. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acid were mixed in 1:3 ratio).

Estimation of glutathione peroxidase (GPx) (EC.1.11.1.9)

GPx was measured by the method described by Rotruck *et al.*^[19] To 0.2 ml tris buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide, and 0.5 ml of tissue homogenate (tris buffer 0.4 M, pH 7.0) were added. To the mixture, 0.2 ml of glutathione (GSH) followed by 0.1 ml of H₂O₂ was added. The contents were mixed well and incubated at 37°C for 10 min, along with a control containing all reagents except tissue homogenate. After 10 min, the reaction was arrested by the addition of 0.5 ml of 10% TCA and centrifuged. The activity was expressed as mg of GSH consumed/min/mg protein.

Estimation of glutathione-S-transferase (GST) (EC.2.5.1.18)

GST activity was assayed spectrophotometrically at 340 nm by the method of Habig *et al.*^[20] The reaction mixture contained an aliquot of 0.1M potassium phosphate buffer pH 7.4, 100 mM GSH, and 100 mM CDNB, which was used as a substrate. The enzymatic activity was expressed as nmol CDNB conjugated/min/mg protein.

Estimation of ascorbic acid

Serum ascorbic acid was estimated by the method of Roe and Kuether.^[21] To 0.5 ml of serum, 1.5 ml of 6% trichloroacetic acid was added and allowed to stand for 5 min and centrifuged. The supernatant was removed and 0.3 g acid washed norit was added, shaken vigorously, and filtered to convert ascorbic acid to dehydroascorbic acid. 2 ml of the filtrate was taken and 0.5 ml of DNPH was added, stoppered and placed in water bath at 37°C for exactly 3 h. After incubation, the tubes were placed in ice-cold water, and 2.5 ml of 85% sulfuric acid was added drop by drop. The contents of the tube were mixed well and allowed to stand at room temperature for 30 min. The color reaction was measured spectrophotometrically at 540 nm. The values of serum ascorbic acid were expressed as mg/dl.

Estimation of α -tocopherol

Serum α -tocopherol was estimated by the method of Baker *et al.*^[22] To 0.1 ml of serum, 1.6 ml of ethanol was added, mixed, and centrifuged. The supernatant was evaporated and

2.0 ml of petroleum ether, 0.2 ml of 2,2'-dipyridyl solution, and 0.2 ml of ferric chloride solution were added, mixed well, and kept in dark for 5 min. An intense red was developed. 0.4 ml of butanol was added to all tubes and mixed well. The color in the layer was read at 520 nm. Standard tocopherol in the range of 10-100 Mg was taken and tested similarly along with reagent blank. The values of serum α -tocopherol were expressed as mg/dl.

Bronchoalveolar Lavage (Fluid) Collection

Immediately after being sacrificed, right lung of mice was lavaged by instillation with 20 ml phosphate buffer saline. The procedure was repeated thrice before lavage fluid was pooled in a heparinized tube and centrifuged at 300 \times g for 10 min at 4°C. Cells were resuspended in 1.0 ml PBS and stained with 0.1% trypan blue.^[23]

Statistical Analysis

Statistical analysis was performed by one-way analysis of variance followed by Fischer's LSD *post-hoc* test using Software Package for the Social Science software package version 12.0. Results were expressed as a mean \pm standard deviation for six mice in each group. $P < 0.05$ was considered significant.

RESULTS

To study the CS exposure in diabetic mice, the levels of serum insulin were significantly depleted in Group-II, III, and IV when compared to that of the control group. The higher decrement was recorded diabetic animals exposed to CS [Table 1]. The levels of TBARS and activity levels of SOD, CAT, and GPx in lungs of control and experimental animals have been summarized in Table 2. The levels of TBARS and activity levels of GST were observed to have significantly higher when compared to that of control, the SOD, CAT, and GPX activities in the lung tissues were decreased significantly in mice exposed to *in vivo* CS. The levels of plasma non-enzymatic antioxidants, *viz.*, vitamin-C and vitamin-E were significantly decreased in CS-exposed animals when compared to that of control; results are shown in Table 3. The serum insulin levels were recorded a significantly depletion in experimental groups when compared to that of control mice. The STZ evoked diabetic mice were exposed to normal air (Sham exposure) and another hand a batch of diabetic mice was exposed to CS. The levels of TBARS and activity levels of GST the lung tissue were observed to have

Table 1: Serum insulin levels in normal and experimental animals

Parameters	Group I	Group II	Group III	Group IV
Insulin (μ mL)	17.20 \pm 1.62	12.83 \pm 1.10*	12.15 \pm 1.48*	10.83 \pm 0.83*

Data are expressed as mean \pm SD of 6 individual observations. Statistical significance * $P < 0.001$. SD: Standard deviation

significantly increased in the diabetic mice (Sham group). However, a higher increment of the above parameters were recorded in the diabetic mice exposed to CS. The antioxidant enzymes, *viz.*, SOD, CAT, and GPx activity levels in the lung tissue were observed to have significantly decreased in the sham-exposed diabetic mice, whereas a significant higher decrease activity levels these enzymes were recorded in the CS-exposed diabetic animal, results are shown in Table 2. Similar trends were recorded that of serum vitamins E and C whose levels were shown to have depleted in diabetic animals with a higher depletion of the vitamins in CS-exposed animal group; results are shown in Table 3.

The BAL study divulges significant hallmarks of apoptosis such as cell shrinkage, cellular surface smoothing, nuclear compaction, and chromatin condensation when compared to that of control mice. The studies on the BAL divulge AM apoptosis in the diabetic mice exposed to CS when compared to that of sham-exposed animals. The AM apoptosis was a significantly higher increment in our study in CS+ hyperglycemia mice when compared to that of control groups (Plates 1-4).

DISCUSSION

Oxidative stress results from an oxidant-antioxidant imbalance: An excess of oxidants and/or a depletion of antioxidants. In this study, the TBARS levels, a measure of lipid peroxidation were determined in the lung tissue. The increased levels of TBARS in CS-exposed animals envisaged the increased levels of lipid peroxidation. The tissue antioxidant SOD, which is primary enzymatic defense in the lungs against the damaging effects of O_2^- , by converting O_2^- into H_2O_2 , which is a substrate for CAT

and GPx. If SOD activity is inadequate, O_2^- can interact with NO to form peroxynitrite ($ONOO^-$) which can react to form the potent OH and nitrogen dioxide (NO_2^-) radicals, which are highly damaging to cell proteins, lipids, and DNA.^[24] Oxidative stress was divulging in the STZ-induced hyperglycemic animals. This might be due to high levels of glucose concentration in circulatory blood. Glucose evoked oxidative damage on the endothelial cells is a characteristic feature in diabetes. The cellular oxidative injury is caused due to the generation of reactive oxygen and nitrogen species which served as important key factors in the pathogenesis of chronic diabetic complications.

Cosentino *et al.*^[25] demonstrated that in human tissue prolonged exposure to high glucose leads to increased production of nitric oxide (NO) and a marked increase of O_2^- generations and the same time due to the presence of high glucose in circulation there is a focused and a concentrated production of nitric oxide and O_2^- oxygen free radicals molecules that would react to produce peroxynitrite, a potent, long living oxidant.^[26,27] Peroxynitrite can inhibit electron transport at the mitochondrial level which oxidizes sulfhydryl groups in proteins and initiates lipid peroxidation affecting many signal transduction pathways.^[28-30]

The oxidative stress glucose toxicity causes activation of various transcription factors and leads to the production of vasoactive factors, and extracellular matrix proteins, which are important features of chronic diabetic complications.^[31] The results of this study where the elevated levels of the TBARS, GST and depleted activity levels of SOD, CAT, and GPx in STZ-induced diabetic animals divulge the oxidative stress. These results agree with literature that evoked

Table 2: Diabetic mice exposed to CS-levels of TBARS, SOD, CAT, GPx, and GST in the lung tissue of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV
TBARS (nm/100 g tissue)	21.12±0.61	55.77±3.40*	75.13±9.81*	100.73±11.81*
SOD (Unit 1/mg protein)	2.03±0.68	0.39±0.12*	0.37±0.12*	0.34±0.32*
CAT (Unit 2/min/mg protein)	10.33±1.32	5.70±0.88*	5.13±0.43*	4.93±0.54*
GPx (Unit 3/mg protein)	12.79±1.66	8.26±1.73*	7.87±1.41*	7.70±0.41*
GST (Unit 4/min/mg protein)	3.97±0.63	6.40±1.64*	8.17±1.74*	8.47±1.44*

Data are expressed as mean±SD of 6 individual observations. Statistical significance * P <0.001. SOD Unit 1: One unit of activity was taken as the enzymes reaction which gives 50% inhibition of NBT reduction in 1 min. CAT Unit 2: μ moles of hydrogen peroxide consumed per minute. GPx Unit 3: μ g of glutathione consumed per minute. GST Unit 4: μ moles of CDNB - GSH conjugate formed per minute. SD: Standard deviation, CS: Cigarette smoke, TBARS: Thiobarbituric acid reactive substances, SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathione peroxidase, GST: Glutathione-S-transferase

Table 3: Diabetic mice exposed to CS - levels of non-enzymatic antioxidants vitamins E and C in serum

Parameters	Group I	Group II	Group III	Group IV
Vitamin C (mg/dl)	0.70±0.39	0.20±0.01*	0.26±0.11*	0.18±0.17*
Vitamin E (mg/dl)	11.05±0.63	3.46±0.48*	3.61±1.26*	3.21±1.26*

Data are expressed as mean±SD of 6 individual observations. Statistical significance * P <0.001. SD: Standard deviation, CS: Cigarette smoke

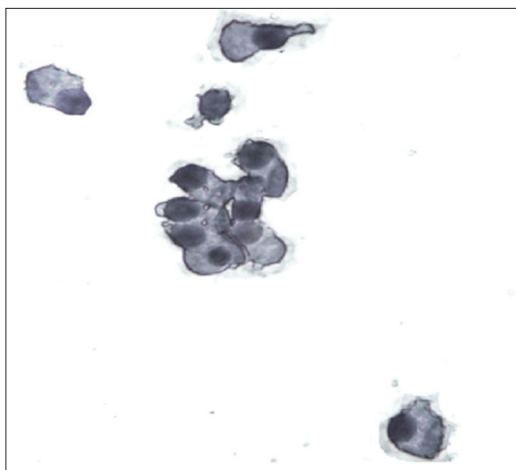


Plate 1: Normal mice exposed to ambient air (normal appearance cells of in bronchoalveolar lavage fluid)

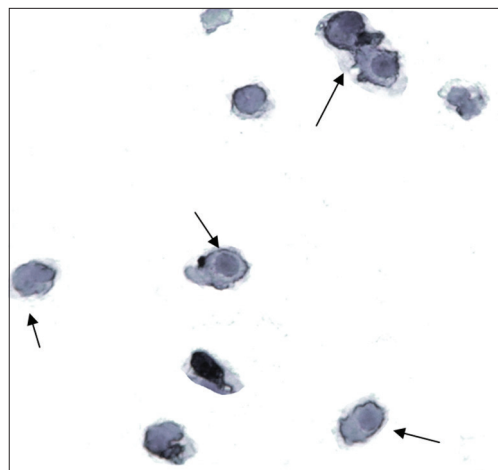


Plate 3: Diabetic mice exposed (sham) to air showing normal animal

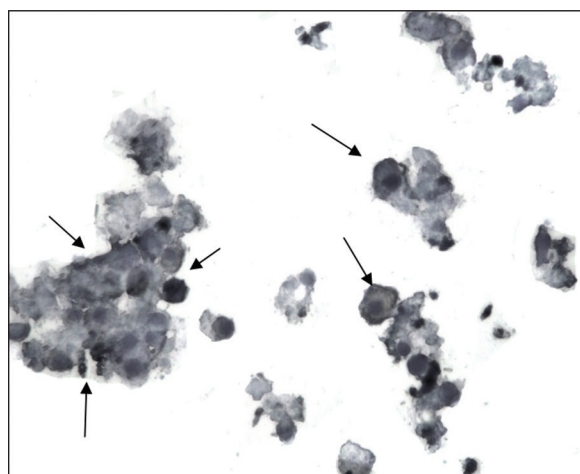


Plate 2: Mice exposed to cigarette smoke; black arrows: Apoptotic cells and larger round cells with eccentric nuclei (alveolar macrophages)

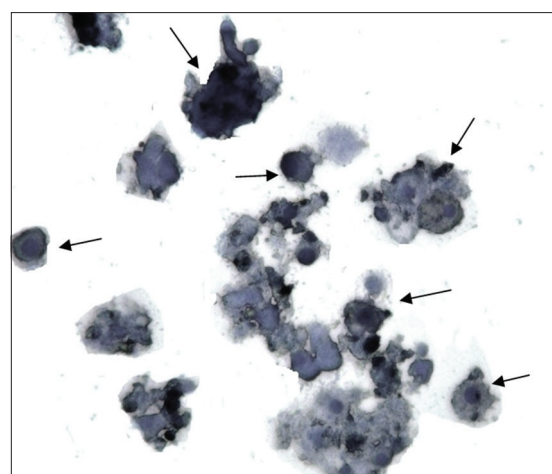


Plate 4: Diabetic mice exposed to cigarette smoke (black arrows): Apoptosis and cell shrinkage

oxidative stress of diabetic animals.^[32] However, the STZ-induced diabetic animals when exposed to CS divulge further indication of oxidative stress. The augmented increment in the levels of TBARS, GST and decreased activity levels of SOD, CAT, and GPX divulge enhanced oxidative stress in the STZ evoked diabetic animals. This might be due to exposure to CS, where the increased free radicals availability might have shifted the oxidants oblique antioxidants balance and thereby initiating oxidative damage similar observations were recorded elsewhere. The non-enzymatic antioxidants, *viz.*, Vitamins E and C depleted. This might be due to increased utilization show has to compact in the increased generation of ROS. Studies on bronchoalveolar lavage depicted increased recruitment of macrophage and increased apoptosis of the same. The results of this study envisaged inflict further infliction of oxidative stress in the diabetic animals exposed to CS, which might be lead to irreparable tissue damage. In conclusion, oxidative stress was evoked by sidestream CS and hyperglycemia and both in combination enhanced lipid peroxidation.

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