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SYNERGISTIC EFFECT OF VITAMINS E AND C ON OXIDATIVE STRESS INDUCED BY SIDE STREAM CIGARETTE SMOKE AND HYPERGLYCEMIA IN ALBINO MICE

M. Akkulu Naidu[1*], Thirunavukkarasu Thirumalai[2], Gangapillai Arumugam[2] M. Akkulu Naidu[3],

Running Title: supplementation of vitamins E and C effect on diabetic mice exposed to cigarette smoke.

[1*]Department of Pharmaceutics, B R Nahata College of Pharmacy, Mhow-Neemuch Road, Mandsaur-458001, Madhya Pradesh, India

[2]P.G, Department of Zoology, Muthurangam Government Arts College, Otteri, Vellore, Tamil Nadu, India-632002.

[3]Department of Biochemistry, Kanchi Shri Krishna College of Arts and Science,Kilambi, Kanchipuram, Tamil Nadu, India-631551.

*****Corresponding Author:
Dr. M. Akkulu Naidu

Department of Pharmaceutics, B R Nahata College of Pharmacy, Mhow-Neemuch Road, Mandsaur-458001, Madhya Pradesh, India
Email: manaidupharmacy@gmail.com
Mobile No: +91-9406674035

Abstract

To evaluate the effects of side stream cigarette smoke exposed to diabetic mice. The mice were exposed to CS of two cigarettes for 15 minutes, twice daily six day a week for 16 weeks. After the protocol, 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. The serum insulin levels were recorded a significant depletion in group-II, VI and VII when compared to that of control animals. The levels of thiobarbituric acid reactive substances (TBARS) and activity levels of glutathione-s-transferase (GST) were significantly increased in experimental groups when compared with control groups. After supplementation of vitamin E and C to the mice group were significantly depleted in Group-V. The activity level of enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were significantly depleted in the experimental animals when compared to that of normal animal. The supplementation of vitamin E & C to the mice was after the schedule of CS exposure, results revealed increased recoup from CS induced oxidative stress, with respect to the activity levels of SOD, CAT and GPx. Supplementation of vitamins E and C appears to be therapeutic in the reinforcement of antioxidant systems that would protect against inducing oxidative stress during hyperglycemia and exposure to cigarette smoke.

Keywords: Cigarette smoke, Reactive oxygen species, hyperglycemia.

1. Introduction

Cigarette smoking is a serious health problem and the most important avoidable causes of death in the world [1]. Smoking has been strongly implicated as a risk factor for chronic obstructive pulmonary disease (COPD), cancer and atherosclerosis, etc [2, 3]. The World Health Organization (WHO) predicts that deaths due to tobacco in India may exceed 1.5 million annually by 2020 [4]. Cigarette smoke contains high concentrations of two different populations of free radicals, one in the tar component and the other in the gas component phase of smoke [5]. About 7000 constituents of mainstream cigarette smoke have been identified [6], thus tobacco smoke causes a mixed

oxidative challenge to the target cells. CS contains high concentrations of reactive oxygen species (ROS) [7]. The major ROS in CS are superoxide anion (O_2^-), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and nitric oxide (NO) [8]. Oxidative stress was induced by side stream cigarette smoke and hyperglycemia and both in combination enhanced lipid peroxidation. Diabetes along with cigarette smoke exposure enhanced the generation of oxygen free radicals [9]. High blood glucose levels during hyperglycemia induced oxidative stress by production of nitric oxide (NO) and marker increased of superoxide anion generation, leading to adverse effects on lung tissues and organs [10]. On the other hand the free radicals and oxidants in cigarette smoke are responsible for lipid peroxidation. The massive surface area makes the lung a target organ for oxidative stress due to cigarette smoke. The augmented levels of oxidative stress leading to enhanced lipid peroxidation recorded in the diabetic mice exposed to cigarette smoke might be due to an add-on effect on oxidative stress of both hyperglycemia and cigarette smoke exposure as well [11]. The supplementation studies of vitamin E, a liphophilic antioxidant, transfers its phenolic hydrogen to a peroxide free radical of peroxidized polyunsaturated fatty acids, thereby breaking the radical chain reactions and averting the peroxidation of membrane lipids. Vitamin-C, a hydrophilic antioxidant, has the ability to sequester the singlet oxygen radical [12], stabilize the hydroxyl radicals and regenerate reduced vitamin-E back to the active state. This synergistic and dynamic effect of vitamins E and C has been studied on oxidative stress. Taking the cue of the results obtained the restorative effects of vitamin E and C on the diabetic mice exposed cigarette smoke has been undertaken in this research.

2. Materials and methods

Chemicals and Reagents

Thiobarbituric acid, reduced glutathione, NADH, ascorbic acid, trichloro acetic acid and α-tocopherol were obtained from sigma chemical company, St Louis, MO, USA. All other chemicals and reagents used were of analytical grade with highest purity and obtained from Glaxo Laboratories (P) Ltd., Mumbai., India

Experimental Animals

Adult male albino mice of Wistar strain weighing around 25-30g were purchased from Tamilnadu Veterinary and Animal Sciences University, Chennai, India. The animals were kept in polypropylene cages (three in each cage) at an ambient temperature of 25±20 C and 55-65% relative humidity. A 12±1 hr light and dark schedule was 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.

Cigarette smoke 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 side stream cigarette smoke into the inhalation chamber sized 30cm (length) ×20cm (width) ×25cm (height). A burning cigarette was introduced through one hole and fixed to a holder and air with a pressure of 0.4 kg/cm2 was passed through the other. The 15 minutes 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 intra peritoneal injection of freshly prepared solution of streptozotocin (STZ) (Sigma,USA) 200mg/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 mg/dl on the third day STZ injection.

Experimental Protocol

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

Group I: Mice (control) were exposed to ambient air. Group II: Mice (experiment) exposed to cigarette smoke. Group III: Cigarette smoke exposure along with saline (0.2 ml) and olive (0.2 ml) supplementation (Placebo). Group IV: Cigarette smoke exposure along with vitamin E (50 mg/kg diluted in 0.2 ml of olive oil/day) and C supplementation (50 mg/kg diluted in 0.2 ml of saline/day). Group V: Cigarette smoke exposure later with vitamin E (50 mg/kg diluted in 0.2 ml of olive oil/day) and C supplementation (50 mg/kg diluted in 0.2 ml of saline/day). Placebo or vitamins E and C supplements were administered by intragastric administration. Group VI: Diabetic mice exposed to air (sham) for 16 weeks. Group VII: Diabetic mice exposed to cigarette smoke for 16 weeks. Group VIII: Diabetic mice exposed to cigarette smoke along with vitamin E (50 mg/kg diluted in 0.2 ml of saline/day) and C supplementation (50 mg/kg diluted in 0.2 ml of saline/day) orally by using intragastric tubes for 16 weeks.

The animals were exposed to side-stream CS of two cigarette for 15 minutes, twice daily six 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 (35mg/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 estimation.

Estimation of Insulin

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

Estimation of lipid peroxidation

Estimation of Thiobarbituric acid reactive substances (TBARS)

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

Estimation of superoxide dismutase (EC 1.15.1.1)

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

Estimation of catalase (EC.1.11.1.6)

Catalase 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 H_2O_2. 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 (EC.1.11.1.9)

Glutathione peroxidase was measured by the method described by Rotruck *et al*. [19]. To 0.2 ml Tris buffer, 0.2 ml of EDTA, 0.1ml of sodium azide and 0.5ml of tissue homogenate (Tris buffer 0.4M, pH 7.0) were added. To the mixture, 0.2ml of GSH followed by 0.1ml of H_2O_2 was added. The contents were mixed well and incubated at 37oC for 10 minutes, along with a control containing all reagents except tissue homogenate. After 10 minutes, 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 (EC.2.5.1.18)

Glutathione-S-transferase (GST) activity was assayed spectrophotometrically at 340nm by the method of Habig *et al*. [20]. The reaction mixture contained an aliquot of 0.1M potassium phosphate buffer pH 7.4, 100mM GSH and100mM 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 0.5ml of serum 1.5 ml of 6% trichloroacetic acid was added and allowed to stand for 5 minutes 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. 2ml of the filtrate was taken and 0.5ml of DNPH was added, stoppered and placed in water bath at 37^{o}C for exactly 3 hrs. After incubation the tubes were placed in ice cold water and 2.5ml of 85% sulphuric acid was added drop by drop. The contents of the tube were mixed well and allowed to stand at room temperature for 30 minutes. The colour 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 0.1ml of serum, 1.6ml of ethanol was added, mixed and centrifuged. The supernatant was evaporated and 2.0ml 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 minutes. An intense red colour was developed. 0.4 ml of butanol was added to all tubes and mixed well. The colour in the layer was read at 520nm. Standard tocopherol in the range of 10-100 Mg were 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, rig
ht 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 heparinised tube and centrifuged at 300×g for 10 minutes at 4^{o}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 (ANOVA) followed by Fischer's LSD post-hoc test using Software Package for the Social Science (SPSS) software package version 12.0. Results were expressed as mean ± S.D. for six mice in each group. *P* values <0.05 were considered significant.

3. Results:

The levels of TBARS and GST in lung homogenates were found to be significantly higher when compared to that of control; whereas the SOD, CAT and GPx activities in the lung tissues were decreased significantly in mice exposed to *in vivo* cigarette smoke (Figure:1). The levels of serum non-enzymatic antioxidants *viz*. vitamin-C, and vitamin-E were significantly decreased in cigarette smoke exposed animals when compared to that of control (Figure: 2). Oral administration of vitamins E and C (50 mg/body weight kg/day) for 16 weeks along with cigarette smoke exposure showed significant decrease in the levels of TBARS and activity levels of GST with an increase in the activity levels of SOD, CAT and GPx in the lung tissue (Figure: 1). The status of improvement of vitamin E and C in serum was then compared to the CS exposed mice (Figure: 2). Saline and olive oil combined had not showed any significant alternations in the parameters when compared to that of control mice. The Bronchoalveolar macrophage (BAL) study revealed significant hallmarks of apoptosis such as cellular shrinkage, cellular surface smoothing, nuclear compaction and chromatin condensation when compared that of control animals (Plate: 1-4). Alveolar macrophage and vacuoles were enlarged in cigarette smoke exposed mice. Vitamin E and C supplementation group animals have been shown to decrease apoptosis in mice BAL. To study the cigarette smoke exposure in diabetic mice. The levels of serum insulin were significantly depleted in Group-II, VI and VII when compared to that of control group. Higher decrement were recorded a diabetic animals exposed to cigarette smoke (Figure: 3). After treatment group were recorded significantly increased when compared to that of control. The supplementation of vitamins E and C to the diabetic mice during the cigarette smoke exposure recorded oxidative stress indicating lowered levels of tissue TBARS and activity levels of GST with increased activity levels of SOD, CAT and GPx when compared to that of same group void of supplementation with vitamins (Figure:1). Further the plasma levels of vitamins E and C were elevated along with activity levels of the endogenous enzymes involved in the antioxidant defense system (Figure:2).

The bronchoalveolar lavage (BAL) studies revealed a restored effect on macrophage apoptosis in the alveoli with vitamin E and C supplementation (Plates5-7).

4.Discussion:

The oxidant effect of cigarettes is due not only to the high quantity of ROS in the smoke, approximately 10^{14} free radicals per puff but also to endogenous ROS that are produced in the lungs mainly by inflammatory cells in alveoli that spontaneously release ROS [24, 25]. It is likely that CS induces an increase in oxidative stress in alveoli, activating inflammatory reactions leading to emphysema [26, 27]. An imbalance between the production of ROS, free radicals and endogenous antioxidant capacity leads to state of oxidative stress that contributes to the pathogenesis of number of human diseases by damaging lipids, protein and DNA [28]. In general many regenerative diseases generate oxidative damage that increases free radicals and ROS. The suitability of the body to oxidative injury depends largely on its ability to regulate protective free radicals and ROS scavenging systems. Vitamins E and C are well known antioxidants individually, that can inhibit oxidative processes involving lipids and lipoprotein in cell membranes. Studies have shown the presence of oxidative stress during hyperglycemia and endogenous antioxidants enzymes such as SOD, CAT and GPx and molecules such as GSH, vitamins E and C beta carotene scavenging free radicals and increased response of antioxidant defense body to the oxidative stress in the body [29].

In the present study significant rise in TBARS levels, lipid peroxidation products, and significant decrease in the antioxidant enzymes such as SOD, CAT and GPx in the experimental mice was indicative of elevated oxidative stress in cigarette smoke exposed hyperglycemic animal group. These results agree with literature where the elevated level of oxidative stress was recorded in mice lung tissue during STZ induced diabetes (hyperglycemia) [11,30] and exposure to cigarette smoke.

The antioxidant vitamin E and C increase the levels of endogenous antioxidant enzymes such as SOD, which catalyze conversion of superoxide radicals to hydrogen peroxide and GPx, metabolize H_2O_2 to O_2 and reduce GSH which in turn produce free radicals [31].

The vitamins E and C have been known as dietary antioxidants enzymes for long time and can eliminate free radical damage thus inducing apoptosis. However these vitamins also been shown to induced protective effect and preventive apoptosis [32].

The elucidative studies on vitamin E and C interactions revealed the role of ascorbic acid in preventing alpha-tocopherol antioxidant functions [33]. These studies also reported that smokers with low level of ascorbic acid in plasma added faster rates of alpha tocopherol disappearance than smokers with high levels of plasma ascorbic acid concentration suggesting that smokers alpha tocopherol disappearance could be attenuated if smokers plasma ascorbic acid concentrations were increased. The alveolar macrophage apoptosis was significantly increased in our study in CS+hyperglycemia animals when compared to that of control groups. The same was decreased along with supplementation of vitamins E and C.

Conclusion

In this study, it was found that the supplementation of vitamin E and C in combination revealed synergistic effect to reduce the levels of TBARS indicating lowered lipid peroxidation and with increased activity levels of endogenous antioxidant defense enzymes. Therefore administration of vitamins E and C appears to be therapeutic in the reinforcement of antioxidant systems that would prevent and /or protect against inducing oxidative stress during hyperglycemia and exposure to cigarette smoke.

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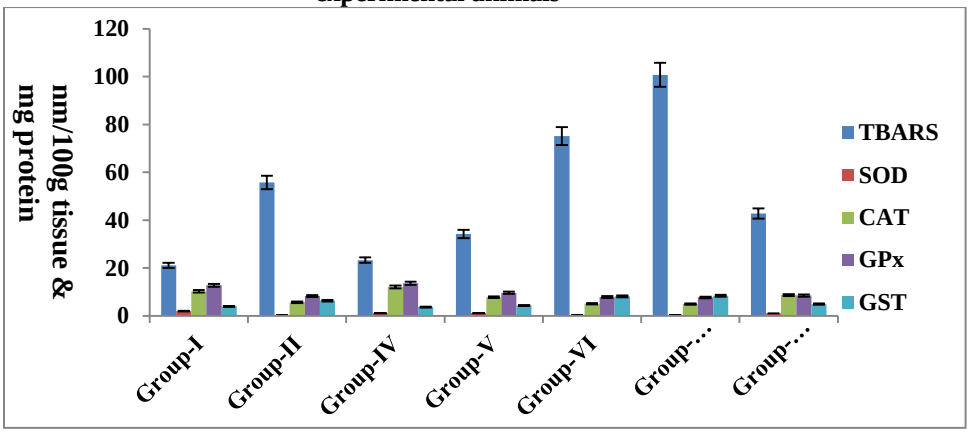
Figure 1: Levels of TBARS, SOD, CAT, GPX, and GST in the lung tissue of control and experimental animals

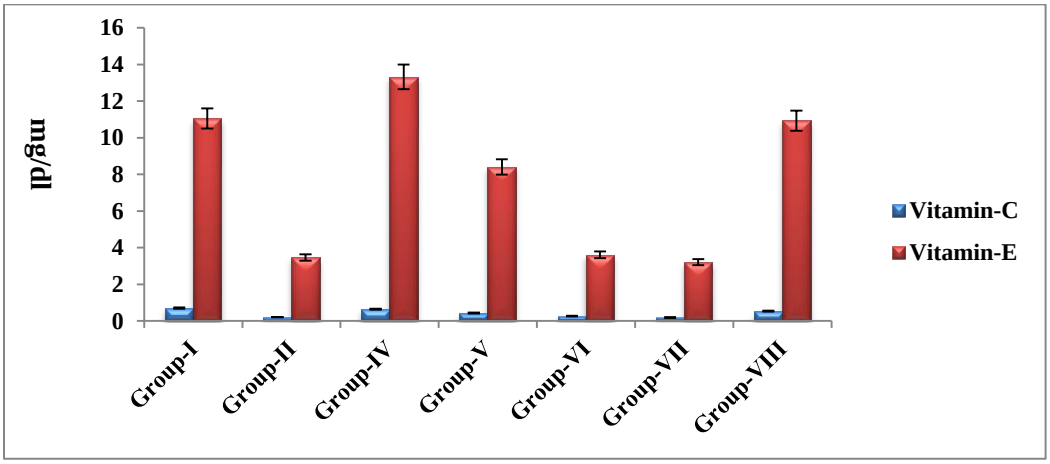


Data are expressed as Mean ±SD of 6 individual observations. Statistical significance P<0.001.

Group-I: Control; Group-II: Cigarette smoke exposed; Group-III: (Placebo) maintained along with group-IV and Group-V; Group-IV: CS exposed along with vitamins E and Supplemented; Group-V: CS exposed later supplemented with vitamins E and C. Group-VI Diabetic mice exposed to air. Group-VII: Diabetic mice exposed to CS. Group-VIII: Diabetic mice exposed to CS supplemented with vitamins E and C.

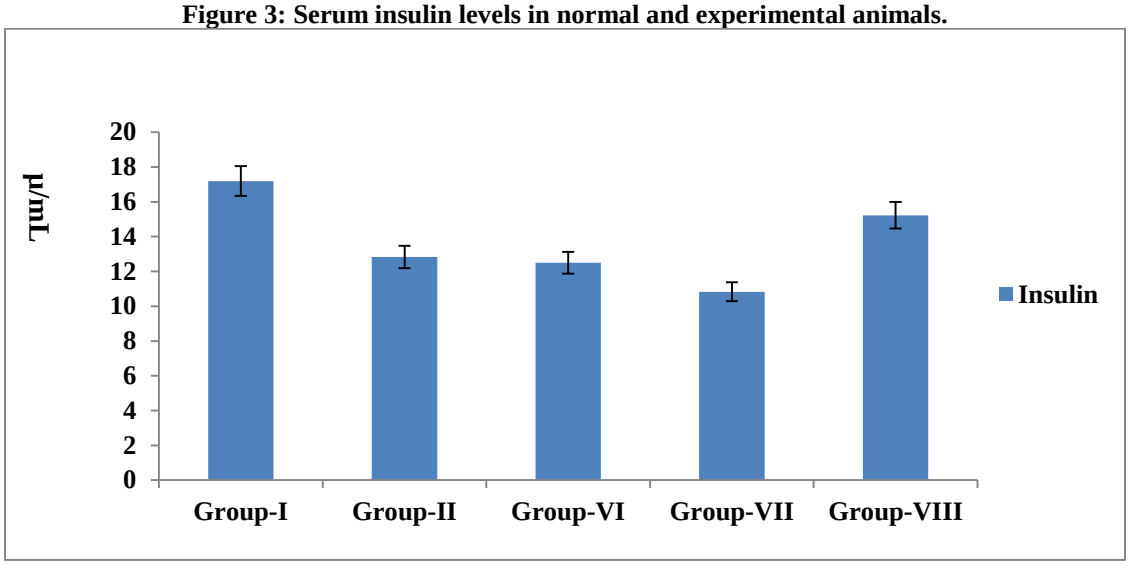
TBARS: Content in tissue, expressed as nm/100 mg.
SOD: U1- One unit of activity was taken as the enzymes reaction which gives 50% inhibition of NBT reduction in one minute.
CAT: U2- µmoles of hydrogen peroxide consumed per minute.
GPx: U3- µg of glutathione consumed per minute.
GST: U4- µmoles of CDNB-GSH conjugate formed per minute.

Figure-2: Levels of non-antioxidant vitamin levels in normal and experimental animals.



Data are expressed as Mean ±SD of 6 individual observations. Statistical significance P<0.001.

Figure 3: Serum insulin levels in normal and experimental animals.



Data are expressed as Mean ±SD of 6 individual observations. Statistical significance P<0.001.

Plates

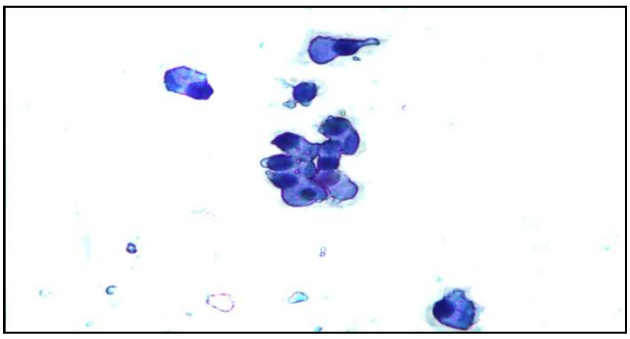


Plate 1: Normal Mice exposed to ambient air (Normal appearance cells of in Bronchoalveolar lavage fluid

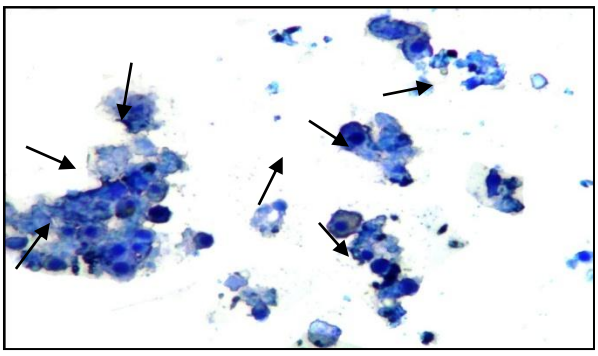


Plate 2: Mice exposed to Cigarette smoke

Black Arrows: Apoptotic cells were enlarged and vacuoles were appeared with eccentric nuclei (alveolar macrophages).

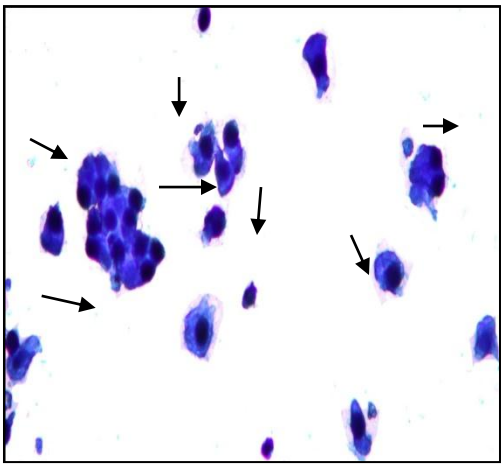


Plate 3: Cigarette smoke exposed mice along with vitamins E & C supplemented. Black arrows: The BAL cells are near to the normal appearance.

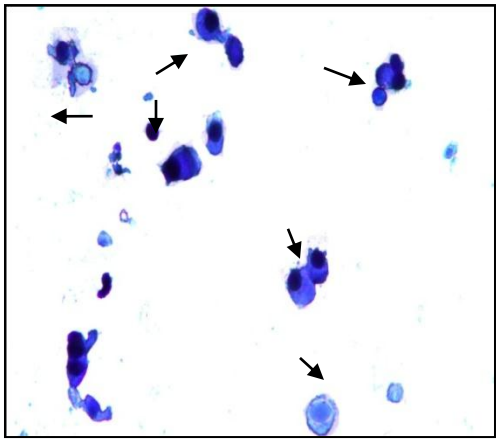


Plate 4: Cigarette smoke exposed mice later with vitamins E & C supplemented. Black arrows: The BAL cells are showing near normal appearance

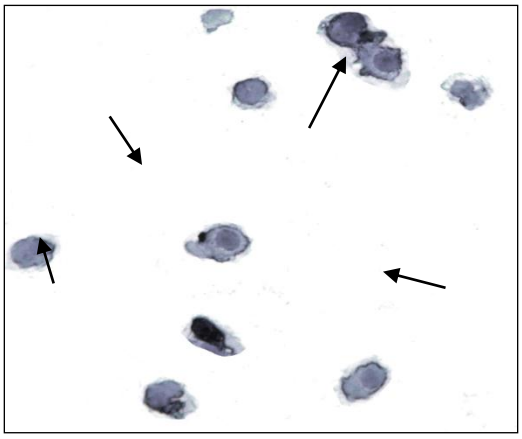


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Plate 5: Diabetic mice exposed (Sham) to air showing normal animal;

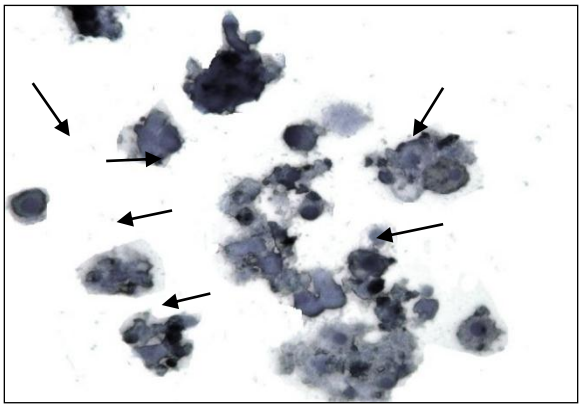

Plate 6: Diabetic mice exposed to cigarette smoke. (Black arrows): Apoptosis and cell shrinkage.

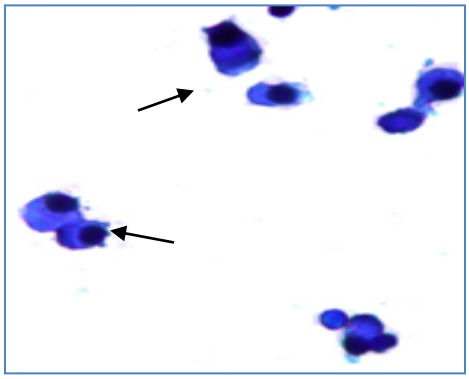

Plate 7: Diabetic mice exposed to cigarette smoke along with vitamins E & C supplemented. The BAL cells are near to that of normal.