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Original Article

In vitro and *in silico* Protective Effects of Ascorbic Acid on Nicotine-treated Human Erythrocytes (Preliminary Studies)

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ABSTRACT

Objectives: Nicotine is one of the most addictive naturally occurring drugs and is commonly found in tobacco products. Tobacco products are one of the most common causes of lung and oral cancer all over the world. As the main organ in contact with nicotine is the blood, our aim in the present study was to confirm its effect on the blood, We also studied the protective role of ascorbic acid on nicotine toxicity. **Materials and Methods:** Different blood toxicity study-related experiments such as Superoxide Dismutase (SOD), catalase, and total antioxidant capacity in erythrocytes were carried out using *in vitro* and *in silico* methods. **Results and Conclusion:** The results demonstrated that nicotine harms red blood cells in the lysis assay as well as the formation of clots in nicotine-treated samples in the blood clotting analysis. Also, the *in silico* method proved the validity of the *in vitro* results. Our research shows that ascorbic acid has protective effects on human blood. It was found that ascorbic acid increases SOD, catalase, and total antioxidant activity. Ascorbic acid also reduced the damage caused by nicotine to Red Blood Cells (RBCs) in the lysis assay, and it showed high protection from the formation of clots that formed when treating the samples with nicotine. The results indicate that the antioxidant ascorbic acid can protect against nicotine-induced hemological damage.

Keywords: Nicotine, *in silico*, *in vitro*, Ascorbic acid, Erythrocytes, Antioxidant.

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INTRODUCTION

Nicotine is considered a naturally toxic substance found in tobacco products.[1] Nicotine is a very addictive drug, and according to a 1988 report on addiction, it is compared to heroin addiction.[2] Nicotine is a hazardous neurotoxin that is extremely deadly and poisonous when consumed.[3] Liquid nicotine, a component of the popular e-cigarette, has recently been linked to an upsurge in poisonings. Because of their small size, children are more susceptible to poisoning.[4] When nicotine enters the body. It causes the adrenal glands to generate adrenaline, which stimulates the body and raises the blood pressure and heart rate, it is also linked with oral and lung cancer; in the United States, 90% of lung cancer is considered to be associated with smoking.[5] Nicotine causes artery narrowing and increases blood flow to the heart, and a high concentration of nicotine can cause arterial wall hardening, increasing the risk of a heart attack.[6] Nicotine shows an adverse effect on blood vessels as well, it causes blood vessels to constrict and lose elasticity, restricting blood flow to organs, and the more blood vessels constrict, the stiffer and less elastic they become. Nicotine remains in the bloodstream for at least three days after smoking.[7] Nicotine raises the number of lymphocytes, leukocytes, and hemoglobin in the blood. It causes an increase in the hemoglobin distribution curve, making hemoglobin levels less useful, which leads to anemia and reduced iron intake. Nicotine raises adrenaline levels, which can lead to blood clotting in blood vessels. Nicotine raises the number of RBCs significantly.[8] Therefore, nicotine raises levels of hormones like adrenaline in the body, which can lead to an increase in blood clot formation and cause an enlarged aorta and atherosclerosis by forming plaque on the artery wall.[9]

High concentrations of nicotine in the body cause an antioxidant imbalance. This imbalance leads to harmful effects on body tissues and organs.[10] The body's antioxidant defense system has both enzymatic and non-enzymatic antioxidants. Glutathione is an important antioxidant whose function is in xenobiotic metabolisms, such as detoxification, which helps in the liver's detoxification process. Antioxidant enzymes such as catalase, peroxidase, and glutathione reduce the free radicals in the cells.[11] According to Fagerstrom,[3] antioxidants protect the blood against nicotine poisoning, and including vitamins E and C, as well as carotenoids, may help protect the red blood cells from free radical damage. Mongi and his team state that ascorbic acid plays a vital role in protecting the blood from free radical-related toxicity.[12] An increased quantity of ascorbic acid intake can elevate the

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antioxidant level up to 30%, which can result in additional protection from free radical-related damage to red blood cells.[13] In the present study, the authors tried to find out the effect of nicotine toxicity on the red blood cells and the role of ascorbic acid in protecting or reducing nicotine-related risk.

MATERIALS AND METHODS

Reagents

We procured tocopherol, ascorbic acid, and pure nicotine from Sigma Aldrich in the USA. Dimethyl Sulphoxide (DMSO) was purchased from Sisco Research Laboratories Pvt. Ltd., (SRL). NaCl, Na_2HPO_4, 30% H_2O_2, and NaH_2PO_4 were from Panreac Quimic. DMSO was used to dissolve antioxidants and nicotine. The phosphate-buffer saline was made by using NaCl, Na_2HPO_4, and NaH_2PO_4 in autoclaved, double distilled water. An assay kit for Superoxide Dismutase (SOD) was purchased from Biodiagnostic Cairo in Egypt.

Blood Sampling

Healthy volunteers donated the 5 of mL blood with informed consent (all the volunteers were in the age range of 20-35 years). The blood samples were collected in heparin coated tubes. All the experiments were performed in triplicates, and mean value was taken for the final calculations.

Osmotic Fragility Assay

An osmotic fragility assay or RBC lysis assay was conducted to study nicotine related osmotic haemolysis. Briefly, 1 mL of heparinised blood was incubated for half an hour at 37°C in absence of any test chemical (control), in presence of 1µg/mL of nicotine, in the presence of 1µg/mL ascorbic acid, and in the presence of both ascorbic acid and nicotine in separate tubes. PBS was diluted in distilled water with concentrations ranging from 10 to 1 g/L. The treated blood was then added to the different concentrations of PBS (with decreasing concentrations) The solution was then slowly mixed by tapping the tubes. All the samples were then incubated for 30 min. All the tubes were centrifuged at 3000 rpm for 5 min. The supernatant was collected, and the Optical Density (OD) at 540nm was recorded. The OD of the collected solutions indicated the degree of RBC lysis. The lysis percentage was calculated by dividing the OD of the supernatant obtained from a particular saline concentration by the OD of the standard (1g/L) represents 100% hemolysis. The lysis percentage was plotted against the concentration of saline solutions to create osmotic fragility curves. The curve yielded the MEF25, MEF50, and MEF75 (mean erythrocyte fragility) values, which are the saline concentrations at which 25%, 50%, and 75% of red blood cells haemolyse (at standard pH and temperature).

Treatment Conditions

To study the effects of nicotine and ascorbic acid together and separately, two different conditions were used, the pre-treatment condition, in which the ascorbic acid was added to the blood 30 min before adding the nicotine to the same sample. In the second condition, both chemicals were added together at the same time (co-treatment).

Statistical Analysis

The results were recorded as standard errors. $P < 0.05$ was considered statistically significant.

Preparation of erythrocyte lysate

For erythrocyte lysate preparation, the erythrocytes were washed four times with 0.9% NaCl, and then the final volume was mentioned up to 2.0 mL by using old sterilized water. The final 25-fold dilution is prepared for the final use.

Superoxide Dismutase Assay

The enzyme Superoxide Dismutase (SOD) activity was carried out using a Superoxide Dismutase Assay Kit (Biodiagnostic).[14] Briefly, the 500 µL/per sample lysate was treated with nicotine, ascorbic acid, and nicotine plus ascorbic acid for 30 min. All the reagents were prepared as per instruction, then Nitroblue Tetrazolium Solution (NBT) and Nicotinamide Adenine Dinucleotide (NADH) (1 mL each) were mixed together immediately before use. 1000 times diluted Phenazine Methosulphate (PMS) was prepared prior to use. 100µL of treated lysate was collected and mixed with the working reagent and PMS (100 µL). The samples were analysed by recording the OD at 560 nm for 5 min.

Catalase Assay

The catalase assay was conducted in erythrocyte lysates.[15,16] In brief, the 100ul treated and untreated samples were transferred into the quartz cuvette, which contained the 2.90 mL H_2O_2 solution (0.036% in 50 mM potassium buffer). The OD was taken for 180 sec, and buffer was used as a blank.

In vitro Lysis assay

Four blood samples were prepared with varying concentrations of ascorbic acid and nicotine, as follows: control, nicotine, ascorbic acid, and a combination of ascorbic acid and nicotine. All the samples were prepared in triplicate and then incubated for 30 min at room temperature. After the incubation the 10 µL of each sample was placed on a glass slide and then stained with methylene Blue. The slides were placed under the microscope and observed for the lysis of RBC.[17]

In vitro Blood clotting Analysis

Four blood samples were collected from the volunteers and placed in heparin treated blood collection tubes (A control

sample, a nicotine-treated sample, an ascorbic acid-treated sample, and a nicotine, and ascorbic acid-treated sample). They were all incubated for 30 min. After that, the samples were placed on slides and dried at room temperature. Then all the slides were observed under a microscope at 100X.

Plasma preparation

The blood was centrifuged for 10 min at 3000 rpm. At 4°C without disturbing the white buffy layer, the top yellow plasma layer was collected.

Total Antioxidant Capacity Assay

The antioxidative capacity is determined by reacting antioxidants in the sample with a predetermined amount of exogenously provided hydrogen peroxide. Antioxidants in the sample remove a portion of the provided hydrogen peroxide. The colorimetric determination of residual H_2O_2 is accomplished through an enzymatic reaction involving the conversion of 3,5,-dichloro-2-hydroxybenzene sulphonate to a colored product.[18] The determination of the antioxidant capacity is performed using a total antioxidant capacity assay kit available commercially. 500 µL of plasma was treated with nicotine, ascorbic acid, and nicotine with ascorbic acid in separate microcentrifuge tubes for 30 min. The working reagent was prepared by adding 1.5 mL of chromogen and 1.5 mL of enzyme – buffer immediately before use. The H_2O_2 was diluted 1,000 times prior to use. After mixing 20µL of treated plasma with 500 µL of substrate (H_2O_2) and incubating for 10 min at 37°C, 500 µL of working reagent was added to the tube. After 5 min absorption was measured at 500 nm.

Table 1: The parameters used in the docking process.

Sl. No.	Parameters	Range
1.	Correlation type	Shape Only
2	FTT mode	3D Fast lite
3	Grid Dimension	0.6
4	Receptor Range	180
5	Ligand Range	180
6	Twist Range	360
7	Distance Range	40

In silico analysis using HEX 8.0

In the current research, bioinformatics tools such as PDB (Protein Data Bank), and software such as Hex 8.0 and Chem-Draw were used. Chem-Draw is a professional tool used mostly by researchers to design and communicate chemical structures. It also aids in the drawing of chemical molecules. The structure of nicotine was drawn using chem-draw software (Li *et al.*; 2004). Hex8.0 was used for docking (Table 1). The protein structures were obtained from the Protein Data Bank (PDB), which provides details about various proteins obtained through X-ray crystallography, NMR, and other techniques.[19]

RESULTS AND DISCUSSION

The mean erythrocyte fragility was used to summarize all of the osmotic fragility assay results (MEF). The MEF levels didn't significantly alter after the pre-treatment (Table 2). However, when both chemicals were applied simultaneously; the co-treatment findings amply demonstrated the protective role of ascorbic acid against nicotine-induced osmotic lysis of red blood cells (Tables 2 and 3). Results from a Superoxide Dismutase (SOD) study showed that nicotine greatly reduced the erythrocytes' SOD activity, but that this harmful effect on SOD was significantly reduced when combined with ascorbic acid (Figure 1). Results of the catalase experiment showed that ascorbic acid neutralized the toxic effects of nicotine on the catalase enzyme (Figure 2). Total antioxidant capacity of human erythrocytes that had been exposed to nicotine, ascorbic acid, or both the findings show that nicotine has a negative impact on red blood cells' overall antioxidant activity, but that ascorbic acid, when combined with nicotine, has a protective effect (Figure 3). Ascorbic acid significantly protected against nicotine-related toxicity, according to an *in vitro* lysis assay (Figure 4). The effects of nicotine on the clotting of blood treated with heparin were also examined in blood samples, and the results showed that the presence of ascorbic acid in nicotine-treated cells inhibits the clotting of red blood cells.[20] (Figure 5). The docking results are presented in Table 4, which enables the receptor molecule to rotate on the Z axis by docking nicotine with various proteins and the Hex program.[21] From the acquired data, nicotine demonstrated a substantial E-total value with superoxide dismutase and heparin.

Table 2: Pre-treatment of ascorbic acid resulting in Mean Erythrocyte Fragility (MEF).

Sl. No.	Sample	MEF25%	MEF50%	MEF75%
1	Control	0.5825±0.08536	0.4425±0.05146	0.2975±0.09146
2	Ascorbic Acid	0.615±0.014142	0.535±0.012583	0.465±0.0125
3	Nicotine	0.735±0.005774	0.565±0.017321	0.51±0.034641
4	Ascorbic Acid +Nicotine	0.66±0.014142	0.5675±0.012583	0.4625±0.012583

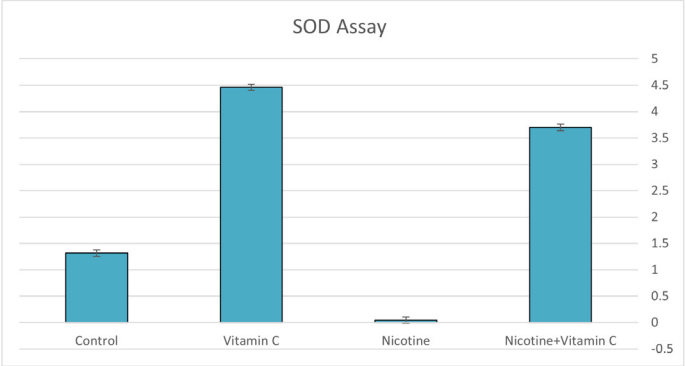

Figure 1: Mean Superoxide Dismutase (SOD) activity (units/mL) of erythrocytes in nicotine treated, and ascorbic acid treated nicotine with ascorbic acid-treated human erythrocyte.

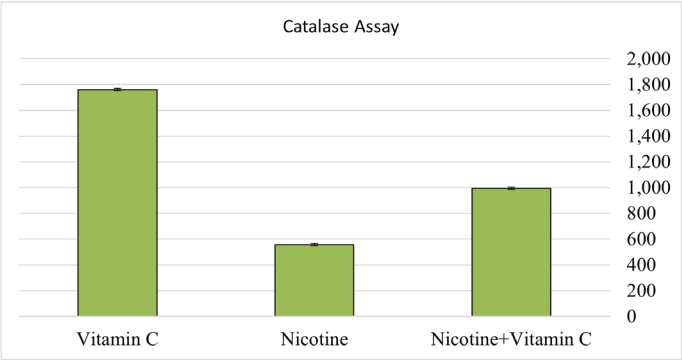

Figure 2: Catalase activity in nicotine treated, ascorbic acid treated, nicotine with ascorbic acid-treated human erythrocyte.

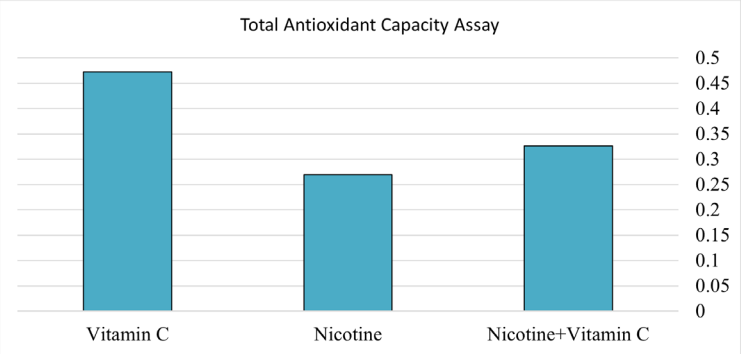

Figure 3: Total Antioxidant Capacity in nicotine treated, ascorbic acid treated, nicotine with ascorbic acid-treated human erythrocyte.

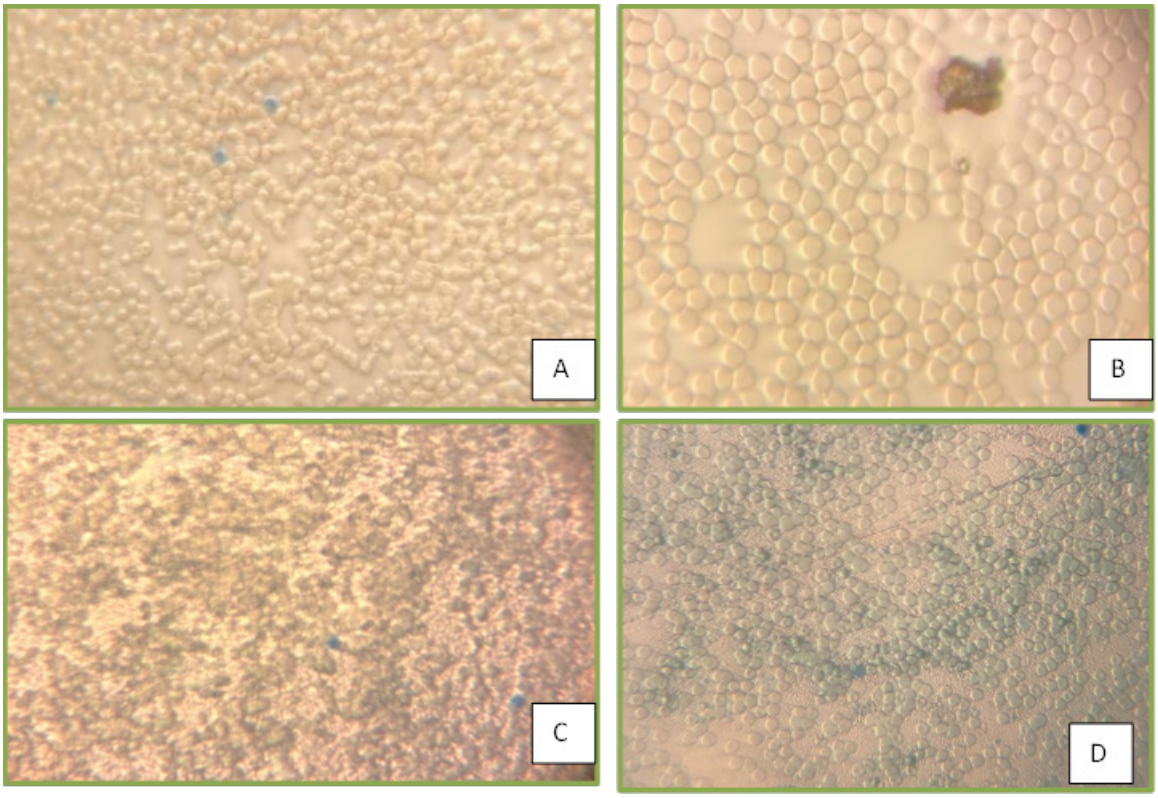

Figure 4: *In vitro* Lysis assay A) Control (B) Ascorbic acid treated (C), Nicotine treated (D) Nicotine + Ascorbic Acid treated.

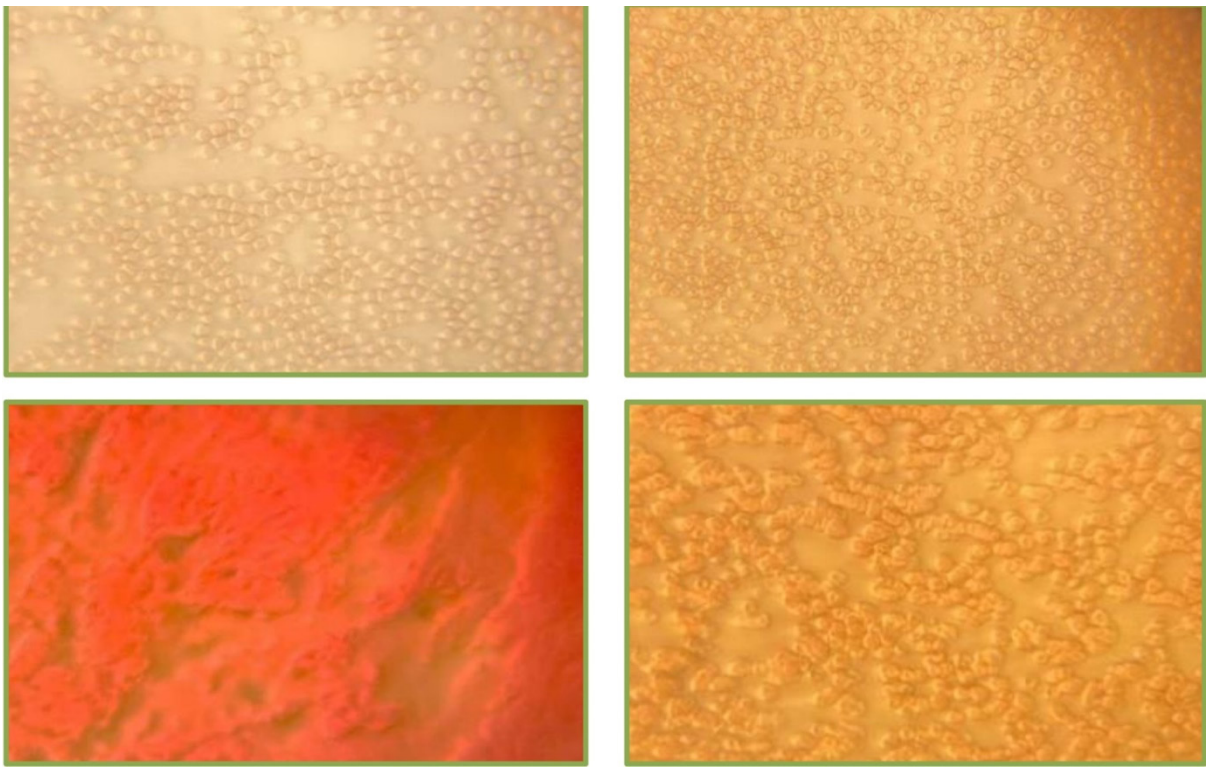

Figure 5: *In vitro* Blood clotting Analysis (A) Control (B) Ascorbic acid treated (C) Nicotine treated (D) Nicotine + Ascorbic Acid treated.

Table 3: Co-treatment of ascorbic acid resulting in Mean Erythrocyte Fragility (MEF).

Sl. No.	Sample	MEF 25%	MEF 50%	MEF 75%
1	Control	0.58±0.052	0.4375±0.996	0.2925±0.996
2	Ascorbic Acid	0.6±0.471405	0.525±0.005774	0.465±0.005774
3	Nicotine	0.721±0.0256	0.59±0.0266	0.51±0.522
4	Ascorbic Acid +Nicotine	0.5725±0.052	0.48±0.066	0.1825±0.002

DISCUSSION

Nicotine is one of the most addictive naturally occurring drugs commonly found in tobacco products. Tobacco products are one of the most common causes of lung and oral cancer all over the world. The effects of nicotine on the body of a human being are varied. Our study proves the toxic effects of nicotine on human blood as it causes oxidative damage to the blood cells. Our data demonstrated that nicotine induces oxidative stress in the human erythrocytes, osmotic lysis, and reductions in the major antioxidant enzymes SOD and catalase. And reduced total antioxidant activity. By completing two experiments designed by us in the laboratory, the lysis assay and the clotting analysis, we were able to demonstrate the severity of nicotine's damage to RBCs, and the clotting formation. According to our results, treating nicotine with ascorbic acid reduced the adverse effects of nicotine. Our research shows that ascorbic acid has protective effects on human blood. The results of the osmotic fragility assay indicated that when the cells were treated with nicotine, the erythrocyte damage caused by osmotic stress was high, but when the cells were treated with nicotine in the presence of ascorbic acid, the effect was decreased. It was found that ascorbic acid increased SOD activity in the superoxide dismutase assay. Nicotine inhibited catalase activity, whereas ascorbic acid increased it. Ascorbic acid demonstrated promising results in terms of increasing total antioxidant activity. Ascorbic acid also reduced the damage caused by nicotine to RBCs in the lysis assay and it showed high protection from the formation of clots that formed when treating the samples with nicotine, as we saw in the previous results.[22] The *in silico* data validate the results of the *in vitro* experiments. The results indicate that all the selected proteins have a very good binding affinity to nicotine, especially Heparin.[23] The results from docking clearly indicated that SOD (E value -162) and heparin (E value -164) have a high affinity for nicotine in comparison with catalase (E value -30.8) and fibrinogen (E value -24.38).

Table 4: Effect of Nicotine over different targets.

Sl. No.	Target protein	E-value
1	Superoxide Dismutase	-162
2	Catalase	-30.80
3	Fibrinogen	-24.38
4	Heparin	-164.91

CONCLUSION

Nicotine is considered to be a very toxic naturally occurring natural product. Nicotine toxicity occurs when nicotine starts to exhibit toxic effects on an individual. Nicotine also has an adverse effect on haematological parameters. Our research shows that nicotine causes acute haemolysis in erythrocytes. Nicotine reduces the activity of SOD and catalase as well as the total antioxidant capacity. Using *in vitro* and *in silico* methods in our research, the results indicate the damaging effect of nicotine can be minimized by treating it with ascorbic acid.

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ABBREVIATIONS

SOD: Superoxide dismutases; **H_2O_2:** Hydrogen peroxide; **PDB:** Protein Data Bank; **MEF:** Mean erythrocyte fragility; **OD:** Optical Density; **RBC:** Red blood cells; **PBS:** Phosphate buffer saline; **NBT:** Nitroblue Tetrazolium Solution; **NADH:** Nicotinamide Adenine Dinucleotide; **PMS:** Phenazine Methosulphate; **NaCl:** Sodium chloride; **Na_2HPO_4:** di-Sodium hydrogen phosphate; **NaH_2PO_4:** sodium dihydrogen phosphate; **DMSO:** Dimethylsulfoxide.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

OA contributed in conducting the experiments and wrote initial draft of manuscript. UF designed the study, provided research materials wrote the final draft and conducted some of the experiments. Both the authors have critically reviewed and approved the final draft.

SUMMARY

- Present study was design to evaluate the nicotine toxicity on human red blood cells and the role of Ascorbic acid.
- Superoxide Dismutase (SOD), catalase, and total antioxidant capacity in erythrocytes were carried out in presence of nicotine and ascorbic acid using *in vitro* and *in silico* methods.
- The results indicated that nicotine causes red blood cells lysis but the ascorbic acid shows protection against nicotine cytotoxicity. Our research shows that ascorbic acid has protective effects on human blood. It was found that ascorbic acid increases SOD, catalase, and total antioxidant activity. Ascorbic acid also reduced the damage caused by nicotine to Red Blood Cells (RBCs) in the lysis assay.

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