Aim: Cigarette smoking is a major risk for developing atherosclerosis; however, the underlying mechanism is poorly understood. This paucity of knowledge is largely attributed to the lack of an animal model; therefore, our efforts were targeted towards establishing cigarette smoke (CS)-induced atherosclerosis in guinea pig. To understand the mechanism, we investigated apoptosis, an event implicated in atherosclerosis, in the aorta of CS-exposed animals. Since a major deleterious effect of CS is oxidative stress, we also examined the effect of vitamin C, an antioxidant, on CS-induced atherosclerosis.

Methods and Results: Guinea pigs on a diet with or without vitamin C supplement were exposed to CS for different time periods. Aortal sections from these animals were examined for atherosclerotic changes by staining with H&E and Oil red O. Atherogenic changes were observed in sections obtained from CS-exposed guinea pigs only. TUNEL assay showed the occurrence of apoptosis in CS-exposed guinea pig aorta. Our results revealed that CS-induced apoptosis could contribute to the progression but not to the initiation of the disease. Immunohistochemical analysis documents that CS-induced apoptosis in aortal sections is mediated at least in part by an increased Bax/Bcl2 ratio. In contrast, CS-exposed guinea pigs fed with vitamin C-supplemented diet exhibit little or no atherogenic changes. This anti-atherosclerotic activity of vitamin C can be attributed partly to its ability to inhibit CS-induced apoptosis and platelet activation.

Conclusion: Exposure of guinea pigs to cigarette smoke causes the development of atherosclerosis, which can be prevented by vitamin C supplement.

Key words; Cigarette smoke, Atherosclerosis, Vitamin C, Apoptosis, Platelets

Introduction

Atherosclerosis is a chronic inflammatory disease characterized by gradual thickening and hardening of arteries. It is caused by the slow build up of plaque on the inner side of arterial walls that leads to the reduction of lumen diameter and restricts blood flow. This results in clinical conditions such as myocardial infarction, a leading cause of death all over the world. The underlying mechanism of atherosclerosis is attributed to endothelial dysfunction, which is induced by oxidative stress originating from several risk factors. Endothelial dysfunction is followed by a series of pathologic events, such as infiltration of inflammatory cells, uptake of oxidized low density lipoprotein (ox-LDL) by macrophages, migration and proliferation of vascular smooth muscle cells (VSMC); formation of vascular lesion with fibrous plaque etc. In the final stage, fibrous plaque ruptures, thereby causing thrombosis.
Epidemiological studies have shown that cigarette smoking is a major cause of atherosclerosis. Despite this, the exact mechanism(s) by which cigarette smoke (CS) induces atherosclerosis is poorly understood. Besides nicotine, CS contains high concentrations of oxidants and free radicals, such as super oxide anions, nitrogen oxides, reactive aldehyde species, carbon monoxide, and hydrogen peroxide, which are considered to be absorbed into the systemic circulation with varying efficiency. They may contribute towards endothelial damage either directly by interacting with the endothelium or by inducing the generation of reactive oxygen species in endothelial and inflammatory cells. CS has also been shown to cause apoptosis, an event leading to cellular death, in cultured endothelial and epithelial cells, as well as in guinea pig lungs; therefore, it is likely that CS causes apoptosis in the aorta, particularly in endothelial cells, which are in direct contact with the circulation. This CS-induced endothelial apoptosis could be a mechanism of endothelial dysfunction.

Previous studies have demonstrated that an aqueous extract of CS (CSE) oxidizes low-density lipoprotein (LDL) into ox-LDL and activates platelets. Such events are considered to be important in the pathogenesis of atherosclerosis; however, all the currently available data were either obtained from epidemiological studies or based on in vitro cell culture studies. Since many cell types and systemic influences contribute towards the onset of atherosclerosis, results obtained from cell culture studies can only convey limited information. Therefore, the current situation demands an animal model for better understanding of CS-mediated events leading to atherosclerosis.

In this study, we directed our efforts towards establishing an animal model of CS-induced atherosclerosis. We report the development of atherosclerosis in the guinea pig aorta after exposing the animals to CS, and have used this model system to probe the underlying mechanism of atherosclerosis. Our results showed the induction of apoptosis by CS in the guinea pig aorta, particularly in its endothelium. Since oxidative stress has been shown to be the major cause of atherosclerosis, and CS is an inducer of oxidative stress, we examined the effect of vitamin C, a known anti-oxidant, on CS-induced development of atherosclerosis. We observed a marked reduction in the development of atherosclerosis caused by CS in animals fed a vitamin C-supplemented diet. To gain insight into the anti-atherosclerotic activity of vitamin C, we also investigated the effect of vitamin C on CS-induced apoptosis and platelet activation.

Materials and Methods
Exposure of Guinea Pigs to Cigarette Smoke (CS)
Three to four-month-old male guinea pigs weighing 350–450 g were used for all experiments. All animal treatment procedures met both the NIH and Institutional Animal Ethics Committee guidelines. Before any experiments, all animals were maintained on a vitamin C-free diet for 7 days to minimize the vitamin C level in plasma and tissues. The diet given to the guinea pigs was similar to that described by King et al., except that wheat flour was replaced with wheat bran. After 7 days of vitamin C deprivation, the guinea pigs were given an oral supplementation of 1 mg vitamin C/day to prevent the onset of scurvy, as it is known that 0.8 mg vitamin C/day is adequate to maintain guinea pigs. Thereafter, the guinea pigs were subjected to either cigarette smoke exposure (3 cigarettes/animal/day with 2 puffs/cigarette) or cigarette smoke exposure and a diet supplemented with vitamin C (5 mg/day) for different time periods as indicated in the figures. At least 3 animals were used for each group. Control guinea pigs were not exposed to CS; instead they were exposed to air and thus this group is hereafter referred to as 0 day-exposed animals. To ensure that all animals were of the same age at the time of sacrifice, the guinea pigs were exposed to CS in such a way that all animals completed their CS-exposure regime on the same day and thus could be sacrificed on the same day. An Indian commercial filter-tipped cigarette (74 mm) (ITC brand) with a tar content of 15 mg and nicotine content of 1 mg was used in all of our experiments. After completion of the experiments, all guinea pigs were sacrificed the next day by diethyl ether inhalation. The aorta was then excised and processed for further analysis.

Histology and Immunohistochemistry
Aortas harvested from guinea pigs were fixed in 4% buffered formalin. Fixed tissue was paraffin embedded, serially sectioned at 5 μM, and stained with hematoxylin and eosin. To determine the presence of lipid, adjacent sections were stained with Oil red O and counterstained with hematoxylin.

Immunohistochemical staining for p53, Bax and Bcl2 was performed on paraffin sections with anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Bax (Santa Cruz) and anti-Bcl2 antibody (Santa Cruz) according to the manufacturer’s protocol. Briefly, sections were deparaffinized in xylene and made permeable by treating with 0.1% Triton X100. Thereafter, antigens were unmasked by heating the sections at 90°C for 10 min in 10 mM citrate buffer, pH 6. The
sections were then allowed to cool at room temperature for 20 min. Next, sections were incubated with the diluted primary antibodies overnight at 4°C. Sections were then washed with PBS and the bound primary antibodies were incubated with FITC-tagged secondary antibody. Fluorescent signals were viewed under a microscope (Olympus IX81).

For Fig. 2, the in situ ROS level was measured by oxidation of 2', 7' dichlorofluorescin diacetate (DCFDA) to highly fluorescent 2', 7'-dichlorofluorescin (DCF). Aortas obtained from animals exposed to CS for 14 days or not were kept frozen and cryo sections were made. Thereafter, sections were incubated with DCFDA for 20 mins at 37°C, washed with PBS and viewed under a microscope (Olympus IX81).

**TUNEL Assay**

The TUNEL assay was carried out on aortic paraffin sections by fluorescein-dUTP labeling using an in situ cell death detection kit (Roche) according to the manufacturer’s protocol. Briefly, prior to the labeling reaction, sections were deparaffinized with xylene. The labeling reaction mixture was added to the sections and incubated for 30 mins at room temperature. Thereafter, sections were washed with PBS and viewed under a microscope (Olympus IX81).

**Platelet Activation Assay**

Platelet activation assays were carried out using a DiaMed Impact R test kit (DiaMed AG, Switzerland) following the manufacturer’s instructions. Briefly, blood sample from a healthy individual, who was not under any medication, was collected in a vial containing 3.2% sodium citrate in 9:1 ratio. After 45-minute incubation at 37°C, the blood was distributed in different aliquots of 130 μL each and mixed with CSE or CSE + vitamin C or ADP, as indicated in Fig. 5. Thereafter, these samples were placed in wells provided with the kit. Next, a cone (also provided with the kit) was placed onto each well and centrifuged to generate shear force. Blood was then discarded, leaving adhered platelets at the bottom of the well. Thereafter, platelets were stained with May-Grünwald dye (supplied with the kit) and the adhesion properties of platelets were analyzed by an Impact-R image analyzer. Results are expressed as % of the well surface covered by aggregates (% SC) as an index of adhesion.

**Results**

**Treatment with vitamin C Reduces CS-Induced Atherosclerosis in Guinea Pigs**

To establish cigarette-induced atherosclerosis in guinea pigs, we exposed guinea pigs to cigarette smoke for different time periods, as indicated in Fig. 1A and observed the changes that occurred in the aorta, a primary site for atherosclerosis, by H&E staining. When guinea pigs were exposed to CS for 7 days (see Methods and Materials), no change was observed as compared to the control (0 day, Fig. 1A); however, with the increase in exposure time (14 days onwards), the aorta clearly exhibited signs of atherogenic changes. At 14 days, visible intima-medial layer thickening was observed in a few aortic sections. Interestingly, after ≥21 days of CS exposure, the endothelial layer was found to be broken in several locations and a massive network of complex plaque was observed within the aortal lumen (Fig. 1A). In contrast, no such structural alteration was observed in sections obtained from guinea pigs exposed only to air (0 day, Fig. 1A). Moreover, some aortal lumens were found to be totally blocked by thrombus formation (Supplementary Fig. 1). Oil red O staining of the adjacent sections confirmed the presence of a high amount of lipid within these structures, indicating an advanced stage of atherosclerosis (Fig. 1A).

CS-induced oxidative stress could play a vital role in this atherosclerotic development. We, therefore, tested the effect of vitamin C, a known antioxidant, on CS-induced atherogenic changes in guinea pig aorta. When guinea pigs were fed a vitamin C (5 mg/day) -supplemented diet along with exposure to CS, no or little structural alterations of the aorta were observed compared to only CS-treated animals (Fig. 1A, 1B). No change in the aorta was observed in guinea pigs that were given vitamin C (5 mg/day) -supplemented food but not exposed to CS (data not shown); therefore, our results clearly demonstrate that CS-induced development of atherogenic changes can be prevented when CS-exposed guinea pigs are given a vitamin C-supplemented diet. To examine whether a dietary supplement of vitamin C reduces CS-induced oxidative stress, we investigated the level of ROS in aortal frozen sections obtained from differentially treated animals by staining with DCFDA, a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the non-fluorescent polar derivative DCFH and is thereby trapped within the cells. In the presence of ROS, DCFH is oxidized to highly fluorescent DCF. Fig. 2 shows that exposure to CS markedly increased the ROS level in endothelial cells; however, dietary supplement of vitamin C prevents this CS-induced increase in the ROS level (Fig. 2). Collectively, these results suggest that oxidative stress caused by CS is a major cause of the atherogenic changes observed in the aorta of CS-exposed guinea pigs, and...
vitamin C can attenuate this pathological event.

**CS-Exposed Guinea Pigs Exhibit Aortal Apoptosis**

To determine if CS exposure results in the induction of apoptosis in the aorta, we performed TUNEL assay on sections adjacent to the sections used in previous H&E staining. Our results showed TUNEL-positive cells indicated by green fluorescence in the aorta of guinea pigs exposed to CS for ≥21 days (Fig. 3). Our results demonstrate that while apoptosis was detected in the endothelial layer of the aorta on Day 21 of CS exposure, it was also detectable within the plaque by Day 28 of exposure to CS. Thus, our results clearly indicate that while apoptosis starts in the aortal endothelium at Day 21, it becomes evident even within the plaque by Day 28.

To gain an insight into the mechanism of CS-induced apoptosis, we examined the level of p53, a protein known to play an important role in apoptosis. We performed immunohistochemical analysis

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**Fig. 1.** Vitamin C prevents CS-induced atherosclerosis in guinea pigs.

A. Exposure to CS causes atherosclerosis in guinea pig aorta. Guinea pigs were exposed to CS for different time periods. A minimum of 3 animals were used for each time point. Aortas obtained from these guinea pigs were fixed in formaldehyde, and paraffin sections were prepared. Thereafter, sections were stained with H&E and Oil red O and viewed under a microscope. B. Vitamin C reverses CS-induced atherosclerosis. Guinea pigs were fed a vitamin C (5 mg/day) -supplemented diet in addition to CS exposure. Paraffin-embedded aortic sections were stained with H&E and Oil red O and viewed under a microscope.
Fig. 2. Vitamin C prevents CS-induced increase in intracellular ROS in guinea pig aortal endothelium.

In situ ROS level was measured by oxidation of 2’, 7’- dichlorofluorescin diacetate (DCFDA) to highly fluorescent 2’, 7’- dichlorofluorescin (DCF). Guinea pigs were exposed either to CS or to air (as control) for 14 days. Three animals were used for each experiment. Aortas obtained from these animals were kept frozen and cryo-sections were made. Thereafter, sections were incubated with DCFDA and fluorescence was viewed under a microscope.

Fig. 3. Exposure to CS causes apoptosis in guinea pig aorta.

Formaldehyde-fixed paraffin-embedded aortal sections, adjacent to the sections used in our previous H&E and Oil red O staining (Fig.1A), were examined for apoptosis by TUNEL assay. Green fluorescence indicates TUNEL-positive cells. Activation of the mitochondria-dependent pathway for apoptosis was tested by immunostaining of the adjacent sections with p53, Bax and Bcl2.
on aorta sections adjacent to sections used in the TUNEL assay (Fig. 3). Our results demonstrate that the level of p53 is increased significantly in the endothelial layer after exposure of the guinea pigs to CS for ≥21 days, which is consistent with the results of the TUNEL assay. To further confirm our results, we also examined the level of pro-apoptotic protein Bax, a target of p53, in the adjacent sections. Similar to elevated p53 levels, an increased level of Bax was observed in the endothelial layer after ≥21 days of CS exposure. Thus, our results clearly demonstrate that exposure to CS stabilized cellular p53, which subsequently increased the level of Bax, a pro-apoptotic protein. We also tested the level of Bcl2 protein in aortal sections, a protein with well-documented anti-apoptotic activity. Consistent with our expectation, significant levels of Bcl2 were observed in the endothelial layer up to Day 14; however, Bcl2 protein was not detected in aortal sections obtained from guinea pigs exposed to CS for ≥21 days and previously shown to be TUNEL positive (Fig. 3). Our results suggest that exposure to CS lowers the Bcl2/Bax ratio and thus induces apoptosis in guinea pig aorta.

Vitamin C Prevents CS-Induced Apoptosis in Guinea Pig Aorta

We observed the attenuation of atherosclerotic development in CS-exposed animals given a vitamin C-supplemented diet (Fig. 1B); therefore, we examined the effect of vitamin C on CS-induced apoptosis by performing the TUNEL assay on paraffin sections adjacent to the sections used in Fig. 1B. When the guinea pigs were fed a vitamin C-supplemented diet, apoptosis was barely observed even after 28 days of CS exposure (Fig. 4). For further confirmation, we analyzed the level of p53 and Bax in the adjacent sections. Consistent with the TUNEL results, our results showed a marked reduction in the level of p53 and Bax in animals fed a vitamin C (5 mg/day) -supplemented diet compared to those without a vitamin C-supplemented diet (Fig. 4). In addition, our results also demonstrate that a dietary vitamin C supplement helps to maintain the Bcl2 level even after ≥21 days of exposure to CS (Fig. 4); therefore, these results indicate that a vitamin C supplement in the diet keeps the Bax/Bcl2 ratio low and prevents apoptosis.

Vitamin C Prevents CS-Induced Activation of Platelets

Previous studies have shown that CS causes platelet activation, an event that contributes greatly to atherogenesis. We examined the effect of vitamin C on CS-induced platelet activation using the Dia

**Fig. 4.** Vitamin C prevents CS-induced apoptosis in guinea pig aorta.

TUNEL assay was carried out on formaldehyde-fixed paraffin-embedded aortal sections that were adjacent to the sections used in Fig. 1B and viewed under a fluorescence microscope. These animals were fed a vitamin C (5 mg /day) -supplemented diet along with exposure to CS. Adjacent sections were immunostained for p53, Bax and Bcl2.

Med Impact R test kit (Switzerland). This assay enables the evaluation of platelet function under close to physiological flow conditions. Human blood was treated with different combinations of cigarette smoke extract (CSE), vitamin C and ADP, as indicated in Fig. 5. ADP is a known platelet activator and thus serves as a positive control. We used 2% CSE since our previous studies indicated that this dose has no cytotoxic effects. Treated blood samples were placed in polystyrene wells and subjected to the Impact R test (see Methods and Materials). Platelet adherence to the well surface was analyzed and quantified by an image
The treatment of blood samples with either ADP or 2% CSE resulted in a marked reduction of platelet adherence compared to untreated blood (Fig. 5). This indicated that the pre-incubation of blood samples with either CSE or ADP, prior to the Impact-R test, led to platelet activation and microaggregate formation in the tube, resulting in reduced platelet adhesion; however, the treatment of blood cells with vitamin C along with CSE reverses the effect of CSE alone (Fig. 5). Treatment with 25 μM vitamin C yields marked improvement of platelet adhesion, which appears similar to normal untreated blood samples (Fig. 5). No further improvement in platelet adhesion was observed using 50 μM vitamin C along with CSE (data not shown). Blood samples treated only with vitamin C exhibit no effect on the adherence of platelets to the well surface (data not shown); therefore, our results clearly demonstrate that vitamin C attenuates CS-induced activation of platelets in a dose-dependent manner.

**Discussion**

In this study we clearly demonstrate the development of atherosclerosis in guinea pigs after exposure to CS. Guinea pig has already been proven to be a good model system for atherosclerosis. Unlike mouse, guinea pig exhibits similarities to human in their cholesterol distribution pattern\(^\text{23}\), which makes it a good model system to study high cholesterol diet-induced atherosclerosis\(^\text{24}\). Besides the accumulation of cholesterol in the arteries, the inflammatory response also contributes towards the development of atherosclerosis. The atherogenic inflammatory process has also been clearly demonstrated in a guinea pig model\(^\text{25}\). In addition, as in human, guinea pig cannot synthesize vitamin C\(^\text{26}\), a known antioxidant, and thus dietary supplementation is the only source of this important vitamin. This is a very important feature, especially for our study, since CS has been shown to cause oxidative stress, which plays a vital role in developing atherosclerosis. For these reasons, we opted for guinea pigs as an animal model of CS-induced atherosclerosis.

To establish the model, guinea pigs were exposed to CS for different time periods and at least three animals were used for each group. Thereafter, we looked at aortal cross sections. Our results showed thickening of the aortic wall in a few sections at the end of 14 days of exposure (Supplementary Table 1); however, when the exposure time was increased to 21 days, we observed atherosclerotic plaques in 60 to 70% of aortal sections, confirmed by Oil red O staining. Similarly, atherosclerotic plaques were observed at the end of 28 days but at a higher frequency than 21-day exposure. In addition, complete blockage of the aortic lumen by thrombus formation was also observed in few sections (Supplementary Fig. 1). During this
study we also noticed that animals started dying if they were exposed to CS for more than 28 days. Since CS has pleiotropic effects and is detrimental to normal physiology, we could not ascertain the exact cause of death; however, in light of our results, it is conceivable that one of the major contributing factors to animal mortality is aortal blockage due to CS-induced atherosclerosis. These observations clearly indicate the development of atherosclerosis in guinea pigs after exposure to CS. Consistent with the results obtained in our cross-sectional study, the lipid profile (an index of atherogenic changes) showed elevated levels of LDL, triglyceride and cholesterol, and reduction of HDL in CS-exposed animals when compared to control animals that were not exposed to CS (Supplementary Table 2). In addition, the plasma carbon monoxide (CO) level, an indicator of exposure to CS, of CS-exposed animals was examined to verify that the only cause of the observed effects was CS. Consistent with our expectations, a higher level of CO was found in the plasma of cigarette-exposed animals (Supplementary Fig. 2). These results clearly demonstrate that exposure to CS causes the development of atherosclerosis in guinea pigs.

A large body of evidence indicates the important role of apoptosis in the development of atherosclerosis. CS has a high oxidant content and is able to generate ROS, which are considered to be pro-apoptotic factors. Moreover, CS has been shown to cause apoptosis in various cultured cell lines, including endothelial cells and lung tissue. In this study we tested the occurrence of apoptosis in aortic sections by TUNEL assay. Sections obtained from guinea pigs exposed to CS for 14 days displayed thickening of the aortic wall; however, no apoptosis was observed in the adjacent sections. We observed apoptosis in aortic sections obtained from guinea pigs exposed to CS for ≥21 days. After 21 days, apoptosis was predominantly found in the endothelial layer; however, with the increase of the exposure period, apoptosis was also observed within the plaque. Consistent with the TUNEL result, stabilization of p53, increased expression of pro-apoptotic factor Bax, and decreased expression of anti-apoptotic factor Bcl2 were found in the same locations as TUNEL-positive cells. Stabilization of p53 and an increased Bax/Bcl2 ratio constitute a mitochondrial function-dependent apoptotic pathway, and thus our results suggest that CS-induced aortal apoptosis involves mitochondrial function, though we cannot exclude the possible involvement of other pathways.

The aortal endothelium is the primary target for many atherogenic risk factors, as it is the first line of defense in the vessel wall. CS-induced endothelial apoptosis definitely impairs this protective function of the endothelium since apoptosis leads to cell loss and thus damages endothelial integrity, which is required for proper functioning of the endothelium; however, our results demonstrate that the initiating event for CS-induced atherogenic changes is not mediated by apoptosis, as we observed neither TUNEL-positive endothelial cells nor the increased expression of pro-apoptotic factor Bax in the endothelium of the aortic section obtained from 14 days CS-exposed guinea pigs, which showed a thickened intima-medial wall, characteristic of atherogenic changes. Therefore, our results showed that exposure to CS causes apoptosis in the guinea pig aorta that may contribute to the rapid progression of atherosclerosis and may help in the formation of thrombus in the aortic lumen, but is not involved in the process that initiates the disease.

Oxidative stress has been considered to play a major role in the development of atherosclerosis. Consistent with this idea, numerous studies have shown an inverse association between atherosclerosis and antioxidant intake. In contrast, several clinical trials found no benefits with vitamin E and vitamin C, which are potent antioxidants, supplementation for cardiovascular disease; however, these clinical trials were not free from limitations. A relatively short period of antioxidant treatment or the treatment of patients with advanced disease may not provide information relevant to disease prevention in healthy individuals. In this context, we tested vitamin C for its ability to prevent CS-induced atherosclerosis in our guinea pig model. We observed that vitamin C treatment prevented changes in the pattern of the lipid profile caused by CS (Supplementary Table 2). Our results clearly demonstrate the prevention of CS-induced atherogenic changes in the aorta of vitamin C-treated guinea pigs. Vitamin C, being an antioxidant, is able to scavenge ROS that are either components of CS or generated by CS. Thus, it may prevent oxidation of LDL, ROS-mediated direct damage to the endothelial layer of the vessel, and oxidative stress-induced signaling pathways that are involved in the initiation and progression of atherosclerosis. We have examined whether vitamin C can prevent CS-induced aortal apoptosis. Consistent with previously published reports showing the inhibition of LPS- and TNF-α-induced endothelial apoptosis by vitamin C in vitro, our results show that vitamin C-treated guinea pigs show no or very little aortic apoptosis after being exposed to CS. Our results demonstrate that treatment with vitamin C reverses CS-induced stabilization of p53 and the subsequent
showed atherogenic changes in the aorta of CS-exposed guinea pigs, and aortal apoptosis in CS-exposed guinea pigs that could greatly contribute to the rapid progression of the disease. We also showed that dietary vitamin C supplement prevented CS-induced platelet activation and thereby provided protection against the development of atherosclerosis.

In conclusion, in this report we present an animal model of CS-induced atherosclerosis. Our results showed atherogenic changes in the aorta of CS-exposed guinea pigs, and aortal apoptosis in CS-exposed guinea pigs that could greatly contribute to the rapid progression of the disease. We also showed that dietary vitamin C supplement prevented CS-induced atherosclerotic changes in the guinea pig aorta. This anti-atherosclerotic activity of vitamin C can be partly attributed to its potential to inhibit CS-induced apoptosis and platelet activation, two key events in the development of atherosclerosis. Therefore, our findings provide insight into some critical atherogenic events triggered by CS in a guinea pig model, which is one of the first of its kind and also establishes the preventive role of vitamin C in the development of the disease. This animal model may prove to be beneficial for further elucidation of the pathomechanism leading to atherosclerosis and to define vital step(s) useful for determining therapeutic strategies.

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Competing Interests

Part of the information is protected by a pending patent application.

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Supplementary Table 1. Development of aortal plaque in CS-exposed guinea pigs supplemented with vitamin C diet or not

<table>
<thead>
<tr>
<th>Vitamin C in diet</th>
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<tr>
<td>CS exposure</td>
<td>0 day (control)</td>
<td>7 days</td>
</tr>
<tr>
<td>Number of animals</td>
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<td>4</td>
</tr>
<tr>
<td>Number of sections studied</td>
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<td>115</td>
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<tr>
<td>Formation of plaque</td>
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*18 sections show thickening of intima-medial layer

Supplementary Table 2. Blood lipid profile in guinea pig that were either exposed or not exposed to CS

<table>
<thead>
<tr>
<th>CS-Exposure</th>
<th>0 Day</th>
<th>21 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>Vitamin C supplement in diet</td>
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<tr>
<td>1 mg/Day</td>
<td>59.3 ± 0.53</td>
<td>120.8 ± 0.6</td>
</tr>
<tr>
<td>5 mg/Day</td>
<td>60.13 ± 0.65</td>
<td>113.53 ± 0.30</td>
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**Supplementary Fig. 1.** Thrombosis occurs in mature stages of atherosclerosis.
H&E-stained paraffin-embedded aortic sections obtained from guinea pigs exposed to CS for 28 days.

**Supplementary Fig. 2.** CS exposure to guinea pigs increases CO level in plasma.
Plasma CO level was measured from blood samples obtained from guinea pigs exposed to CS for 21 days or not.