Vitamin C: A potential saviour against free radical-induced oxidative damage

Indu B. Chatterjee, Chinmay K. Mukhopadhyay and Mrinal K. Ghosh

Dr B. C. Guha Centre for Genetic Engineering and Biotechnology and Department of Biochemistry, University College of Science, Calcutta 700 019, India

Scurvy is the foremost nutritional-deficiency disease that has caused maximum suffering in recorded history. Although research on vitamin C started in 1753, the biochemical functions of the vitamin described so far appear to be nonspecific. This paper is a short review indicating that vitamin C protects mammalian tissues from oxidative damage and that its function is specific. Since oxidative damage is supposed to be a physiological event associated with degenerative diseases and aging, vitamin C appears to be a saviour in protecting us against aging and degenerative diseases.

OXYGEN sustains life, but oxygen is not absolutely friendly. The function of oxygen is to act as a terminal electron acceptor. Usually oxygen receives four electrons on a concerted fashion to produce water, but often it is reduced by single electron to produce oxygen-free radical (O_2^-) and reactive oxygen species (ROS), such as H_2O_2, $OH^{\cdot}$ and ferryl. ROS is highly toxic and extremely damaging to biological systems. If not properly scavenged, it results in oxidative damage such as lipid peroxidation, protein oxidation and DNA damage. Oxidative damage has been implicated in several degenerative diseases including inflammation, arthritis, cataract, atherosclerosis, cardiovascular diseases, cancer as well as aging[1-7]. A simple but effective way to prevent these degenerative diseases would be to prevent oxidative damage. A cell's major defence against free radical-mediated damage includes antioxidants like ascorbic acid, vitamin E and glutathione and enzymes like super-oxide dismutase, catalase and peroxidases. Sometimes ascorbic acid and vitamin E as well as ascorbic acid and glutathione function as partners in defence[8-11]. Previously, we and others have shown that ascorbic acid is a potential scavenger of O_2^-, $^1O_2$, and peroxyl radical[12-15]. In this review, we present a brief account of the discovery of vitamin C, its biosynthesis, and newer insights into its biochemical functions with particular emphasis to indicate that vitamin C (ascorbic acid) is a unique antioxidant and in certain conditions it has a specific role in preventing ROS-mediated oxidative damage of mammalian tissues.

Scurvy and the discovery of vitamin C

Most animals synthesize their own requirement of vitamin C, but a human cannot. Humans are totally dependent upon dietary intake of the vitamin. The knowledge that man cannot produce vitamin C was learnt by experience. But the experience was horrible. During the period of 1500 to 1800 AD, scurvy took a toll of at least two million deaths of sailors[16]. Scurvy also ravaged whole armies and inhabitants of besieged cities. It is perhaps the foremost occupational disease and the nutritional-deficiency disease that has caused most suffering in recorded history[17]. It was the genius of James Lind[18] which banished from the navy the most merciless killer of seamen. In 1753 in his book *A treatise of the scurvy*, Lind pointed out the importance of oranges, lemons and fresh green vegetables in preventing and cure of scurvy. But it took another 175 years to discover the antiscorbutic vitamin by Albert Szent-Györgyi. This discovery was accidental. While extracting and concentrating some redox compound from adrenal glands, Szent-Györgyi isolated some sugar-like crystals about which he was quite ignorant and he called it first 'ignose' [(ig for ignorance and ose for sugar)] and later 'godnose' (God knows)[17]. But the editor of the *Biochemical Journal* objected and he had to give the alternative name hexuronic acid (hex = six). In the same period, Charles Glen King of USA isolated the antiscorbutic factor from lemon juice and named it vitamin C[17]. It showed all the characteristics of Szent-Györgyi's hexuronic acid. It was published in *Science* dated April 1, 1932 entitled 'The chemical nature of vitamin C'. Two weeks later, a note appeared in *Nature* entitled 'Hexuronic acid as the antiscorbutic factor', authored by Svirbely and Szent-Györgyi[17]. But these discoveries were possible only because of the outstanding observation by the Norwegian scientists, A. Holst and his associates. Holst observed that guinea pigs, like humans, were prone to scurvy and could be used for producing experimental scurvy and bioassay of vitamin C. At that time there was quite a flurry as to who should be given the credit for 'discovering vitamin C'. Eventually, Szent-Györgyi received the Nobel Prize for Physiology and Medicine in 1937 in recognition of his discoveries concerning the biological oxidation process with special reference to isolation of vitamin C, but C. G. King, who published five papers and isolated vitamin C, did not. Holst, who made it all possible, died in 1931 without any special honours. Here we see that a scientist, working only for a year in Hopkin's laboratory on a problem with quite

a different objective and without doing a single animal experiment, got the full credit for the discovery of vitamin C. To add to this, in the *Annual Review of Biochemistry* in 1963 (ref. 19), Szent-Györgyi made the comment – 'I was not acquainted with animal tests in this field and the whole problem was for me too glamorous, and vitamins were, to my mind, theoretically uninteresting. Vitamin means that one has to eat it. What one has to eat is the first concern of the chef, not the scientist'.

Biosynthesis of ascorbic acid

While the knowledge of cause and cure of scurvy was clear in 1928–32, the question remained unsolved – How is vitamin C synthesized by most animals and why are humans incapable of producing the vitamin? It took about another thirty years to answer these questions. The most intriguing parts of the problem were the discovery and characterization of two enzymes, namely, aldonolactonase (EC 3.1.1.18, L-gulonolactone hydrolase)[20] and L-gulonolatone oxidase (EC 1.1.3.8, L-gulonolactone : oxygen 2-oxidoreductase) and identification of 2-keto-L-gulonolactone[21–23], an extremely unstable terminal intermediate. Eventually the pathway of biosynthesis of ascorbic acid was completely elucidated by the pioneering work of B. C. Guha (India), C. G. King (USA) and L. W. Mapson (England) and their associates. In animals, ascorbic acid is synthesized from glucose via the glucuronic acid pathway of metabolism[24,25]. In this biosynthetic pathway, L-gulonolactone oxidase is the terminal enzyme. L-gulonolactone oxidase converts L-gulono-1,4-lactone to 2-keto-L-gulonolactone, which is spontaneously converted to ascorbic acid.

Evolution and ascorbic acid biosynthesis

There is a distinct relationship between evolution and the capacity to synthesize ascorbic acid in animals[26–28]. The evolution of L-gulonolactone oxidase (LGO) is species-specific and tissue-specific. LGO is absent in fishes. The enzyme evolved in the kidney of amphibians, resided in the kidney of reptiles, became transferred to the liver of mammals and finally disappeared from the guinea pig, flying mammals, primates including monkeys, apes and humans as well as some highly evolved passeriformes birds[28]. Lack of LGO is the common genetic defect in all these species incapable of synthesizing vitamin C[26,29]. In fact, lack of LGO is an evolutionary loss. Recently, the gene for LGO has been cloned and sequenced by the Japanese group[30–32].

Biochemical functions of vitamin C

Although the pathological syndrome of scurvy was delineated by Lind about 242 years ago, the precise biochemical function of the vitamin remained unclear. One of the reasons is the negative contribution by eminent scientists who had success in one field but were perhaps over-confident in proposing theories in the field of vitamin C which they had not studied in depth in the way they had their original field of work. One single example will suffice to understand this. McCollum, the discoverer of vitamin A, drew the surprising conclusion from his experiments with guinea pigs that it was constipation in the guinea pig, rather than the lack of an unidentified factor in the diet, which was the cause of scurvy[17]. He did not realize that scurvy was a generalized disintegration of all tissues leading to loss of life's total occupation, not to speak only of constipation. In fact, vitamin C had long been neglected. The knowledge that vitamin C is needed for the prevention and cure of scurvy was the cause of ample satisfaction to some people, particularly of the medical profession. Quite a few claims have been made about the biological functions of vitamin C, but most of these are nonspecific[33,34]. Research on vitamin C got a tremendous boost in 1970 and onwards after the publication of a paper in the *Proceedings of National Academy of Science, USA*[35] and a bestseller book, *Vitamin C and the Common Cold*[36] by Linus Pauling. However, Pauling's claim that large doses of vitamin C were beneficial for cold and cancer has not been substantiated by later research. But in spite of the debate, controversy and conflicting results about Pauling's theory, Pauling's message has kept vitamin C research alive. In the five years ending 1981, for instance about 5000 papers were published on vitamin C. But still the precise biochemical function of vitamin C remained controversial. None of the roles of ascorbic acid, described before,[33,34] permits a correlation with the pathologic syndrome found in scurvy, which in reality is a syndrome of generalized tissue disintegration at all levels, both intracellular and extracellular. The generalized tissue disintegration in scurvy is accompanied by subcutaneous and intramuscular haemorrhage in various parts of the body, disruption of collagen being a major cause.

Collagen synthesis

The link between collagen synthesis and vitamin C has dominated our thinking for decades. Collagen is a fibrous connective tissue protein which is abundant in tendons, ligaments, basement membranes, bone matrix and blood vessels. Collagen comprises about one-third of the body protein. The stability of collagen depends upon its triple helix structure, which, in turn depends on the presence of the unique amino acid, hydroxyproline. Ascorbic acid stimulates collagen synthesis by stimulating hydroxylation of proline, which is catalysed by prolyl hydroxylase. Scurvy was thought to be caused due to lack of proper

hydroxylation of proline. However, there is little evidence for direct participation of ascorbic acid in collagen synthesis. The function of ascorbic acid for the stimulation of collagen synthesis is to keep the nonheme iron of prolyl-4-hydroxylase in the active state[37]. During the catalytic reaction, a highly reactive iron–oxygen complex, a ferryl ion, is produced, which subsequently hydroxylates an appropriate proline residue[38]. However, the generation of ferryl ion also proceeds without proline hydroxylation in uncoupled reaction cycles. Ascorbate is utilized as a specific alternative acceptor of the ferryl in these uncoupled reaction cycles. In the absence of ascorbate, prolyl-4-hydroxylase is rapidly inactivated by self-oxidation[38]. It is debatable whether a principal function of ascorbic acid is stimulation of collagen synthesis or prevention of disruption of collagen bundles. Recent evidence from our laboratory indicates that ascorbate protects collagen from ROS-induced oxidative degradation. Moreover, studies both in vivo and in vitro indicate that ascorbic acid prevents ROS-mediated microsomal lipid peroxidation and protein degradation. These have been discussed later in this paper.

Evolutionary significance

The protective role of ascorbic acid against oxidative damage has evolutionary significance. LGO evolved in the amphibians[26]. The class Amphibia emerged and diversified in the Devonian–Permian, when the atmospheric oxygen concentration increased from 15% to 35% (ref. 39). The water oxygen content was about 0.74% (ref. 39). This would indicate that during evolution of the vertebrates in the terrestrial atmosphere, the tetrapods were exposed to an environmental oxygen concentration of about 47 times that of their aquatic ancestors. This extreme hyperoxia might be a cause of extinction of most of the early amphibians[40]. It is conceivable that only those amphibians that acquired a strong defence mechanism against oxygen toxicity survived successfully. It is known that the form of oxygen responsible for toxicity in hyperoxia is O_2^- and its progeny. The enzymatic defence against O_2^- is superoxide dismutase (SOD)[41–43]. In fact, SOD was contingent for the evolution of early aerobic organisms[41]. In the prokaryotes, SOD is induced in hyperoxia. Exposure of E. coli and S. faecalis to 25 atm oxygen elicits induction of SOD synthesis 16–25 times, which correlates acquisition of tolerance towards hyperoxia[41]. However, SOD levels of amphibian tissues did not increase over that of fishes[14]. Instead, there was a burst of LGO activity in those successful amphibians[26,27], that survived the hyperoxia through the late Carboniferous and early Permian. The successful tetrapods started synthesizing high amounts of ascorbic acid apparently to protect the tissues against oxidative damage. In the phylogenetic evolution, the biosynthetic

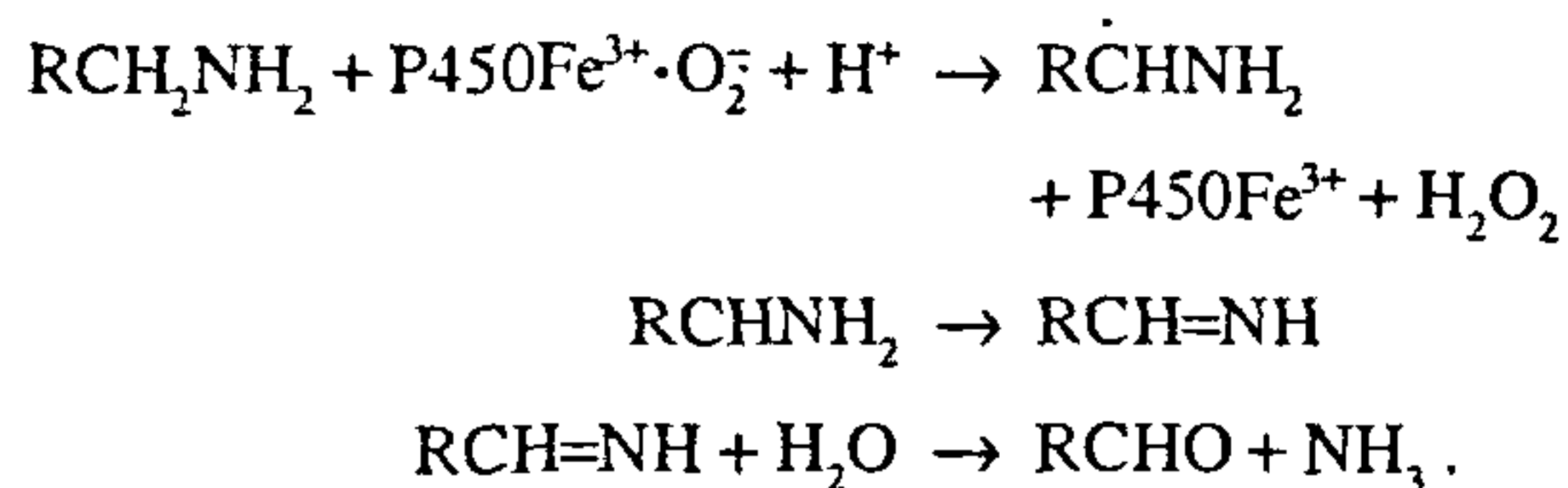capacity continued in the reptiles, most of the mammals and birds.

Subclinical deficiency

Lack of ascorbic acid causes scurvy. So it would appear that if there is no scurvy there is no lack of ascorbic acid. However, scurvy is not a first symptom of lack but a final collapse, a premortal syndrome, and there is a very wide gap between scurvy and full health[44]. Although frank scurvy is rare nowadays, a more incidious condition – the subclinical ascorbic acid deficiency – is common in many parts of the world, particularly involving the nursing mother, infant and elderly. Subclinical deficiency, or marginal ascorbic acid deficiency as it may be called, relates to a state of low tissue level of ascorbic acid, which might cause incipient biochemical changes without showing any apparent clinical symptom. We have recently observed[5,45] that in marginal ascorbic acid deficiency in the guinea pig, oxidative damages such as lipid peroxidation and protein oxidation occur progressively in almost all tissues including liver, lung, heart, kidney, adrenal glands and testes. The oxidative damage occurs despite the presence of adequate levels of other antioxidants like α-tocopherol, glutathione, protein thiols and scavenging enzymes, namely SOD, catalase and glutathione peroxidase[5,45]. Lipid peroxidation and protein changes disappear after ascorbic acid therapy. We have taken the guinea pig as a model animal, because like humans it cannot synthesize ascorbic acid, so the results may be applicable to humans. Particular mention should be made about heart tissue[5]. The levels of antioxidants and free radical scavenging enzymes are normally low in the heart. We have indicated[5] that chronic subclinical ascorbic acid deficiency may produce progressive oxidative damage which in the long run may lead to permanent degenerative vascular diseases and myocardial infarction.

Protection of microsomal membranes against lipid peroxidation and oxidative damage of proteins

Numerous studies[2,46] have been made in vitro for elucidating the mechanism of oxidative damage and its prevention. However, most of these studies require the presence of free iron in the incubation medium, a condition which has apparently no relevance to the in vivo situation. This is because in the normal physiological condition, iron does not exist in the free state but remains tightly bound with proteins. Using a model in vitro system, which is more relevant to the in vivo situation, we have demonstrated[47,48] that NADPH initiates lipid peroxidation and oxidative degradation of microsomal proteins in the absence of free iron. Lipid peroxidation and protein

degradation are mediated by cytochrome P450 and specifically prevented by ascorbic acid. Other scavengers of ROS including SOD, catalse, GSH, vitamin E are ineffective. Ascorbate also prevents O_2^--initiated free iron-independent P450-mediated protein oxidation. Probably the oxidant is a perferryl moiety, namely, P450 $Fe^{3+}\cdot O_2^-$. The oxidative degradation of proteins occurs irrespective of tissues, namely liver, kidney, lung, heart, adrenal gland and brain[47,48]. It is a two-step process: (i) P450Fe$^{3+}\cdot O_2^-$-mediated oxidation of proteins and (ii) rapid proteolytic degradation of the oxidized proteins. Using lysyl residue as a model, a mechanism has been proposed[47] for P450Fe$^{3+}\cdot O_2^-$-mediated protein oxidation as indicated below.

$$RCH_2NH_2 + P450Fe^{3+}\cdot O_2^- + H^+ \rightarrow RCHNH_2$$
$$+ P450Fe^{3+} + H_2O_2$$
$$RCHNH_2 \rightarrow RCH=NH$$
$$RCH=NH + H_2O \rightarrow RCHO + NH_3 \ .$$

Apparently, P450Fe$^{3+}\cdot O_2^-$ abstracts a hydrogen from the side chain amino acid residue producing a carbon-centred radical followed by its conversion to an imino derivative, which then undergoes spontaneous hydrolysis producing an aldehyde derivative of the lysyl residue. Ascorbic acid specifically prevents initiation of protein oxidation by providing an easily donatable hydrogen for abstraction by P450Fe$^{3+}\cdot O_2^-$, thus sparing the side-chain amino acid residue as shown below:

$$P450Fe^{3+}\cdot O_2^- + AH_2 \rightarrow P450Fe^{3+}\cdot O_2^= + AH^{\cdot} + H^+$$
$$AH^{\cdot} + AH^{\cdot} \rightarrow AH_2 + A.$$
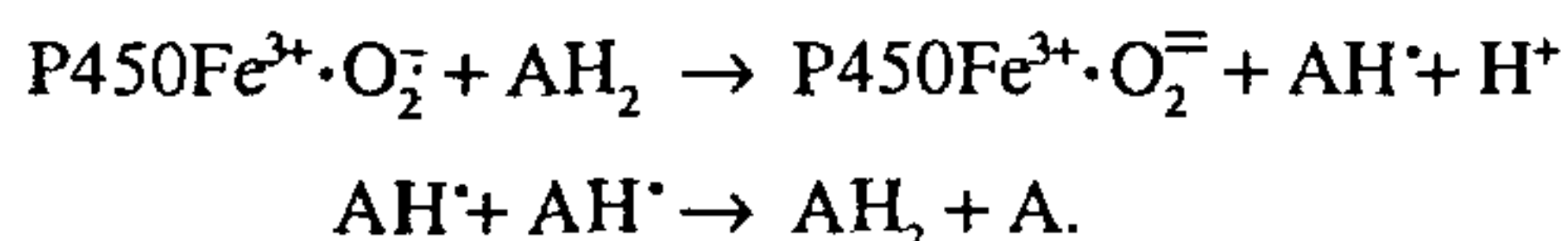
In this process, ascorbate (AH_2) is oxidized to ascorbate-free radical ($AH^{\cdot}$), which apparently decays by disproportionation to AH_2 and dehydroascorbate (A). Once ascorbic acid intercepts the reaction between P450Fe$^{3+}\cdot O_2^-$ and the side chain amino acid residue, the oxidation of the protein is prevented. So, the subsequent proteolytic degradation of the protein is also prevented.

Protection of collagen against oxidative damage

We have also shown that ascorbic acid prevents oxidative degradation of collagen by ROS produced by O_2^- generated from activated macrophages[49]. The extracellular matrix of mammals contains numerous macrophages that undergo oxidative burst during phagocytosis and release in the environment large amounts of O_2^-, redox proteins, like cytochrome b_{558} as well as metalloproteinases. As mentioned above, in the case of microsomal cytochrome P450, collagen is apparently oxidized by a ferryl type of ROS produced from O_2^- and cytochrome b_{558}. The

oxidized collagen subsequently undergoes proteolytic degradation by the metalloproteases. Exposure of collagen fibrils to O_2^- generated by the xanthine–xanthine oxidase system also results in rapid oxidation of collagen[49]. In this case, the collagen oxidation is a function of the protein-bound redox iron of xanthine oxidase (XOD). The reactive oxygen species involved is also probably a ferryl moiety, namely, XOD-Fe$^{3+}\cdot O_2^-$. As observed in the case of microsomal proteins, ascorbic acid apparently prevents collagen oxidation by interacting with the perferryl moiety. Once collagen oxidation is prevented, the subsequent proteolytic degradation of collagen is also prevented. SOD also prevents O_2^--induced collagen breakdown, but in contrast to ascorbic acid which is ubiquitous *in vivo*, the content of SOD in the extracellular fluid is negligible[50,51]. This imparts a specific important role of ascorbic acid for the protection of collagen in the extracellular matrix of mammalian tissues. We consider that an apparently specific function of ascorbic acid is to protect the collagen of the extracellular matrix from oxidative degradation.

Conclusion

The above mentioned results indicate that a potential and apparently specific role of ascorbic acid is to protect the mammalian tissues against oxidative damage both at the intracellular and extracellular levels. We have reported that even in subclinical ascorbic acid deficiency, oxidative damage occurs as evidenced by lipid peroxidation and damage of microsomal proteins[5,45] and that persistent subclinical deficiency results in progressive oxidative damage. We postulate that scurvy symptoms in acute ascorbic acid deficiency is apparently a premortal syndrome of severe oxidative damage, leading to disintegration of tissues. In our present day lifestyle, we are continuously exposed to environmental pollutants such as cigarette smoke, drugs, pesticides, xenobiotics and car exhausts. These pollutants result in increased production of reactive oxygen species, leading to increased oxidative damage such as lipid peroxidation and oxidative degradation of proteins. Since oxidative damage is supposed to be a physiological event associated with various degenerative diseases and aging, we postulate that vitamin C is probably a potential saviour against aging and degenerative diseases.

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