CIGARETTE SMOKE THE KILLER: VITAMIN C THE HEALER*

INDU BHUSAN CHATTERJEE**, SHINJINI GANGULY**, ARUNAVA GHOSH**, ARCHITA DAS**, SANTANU BANERJEE**, NEEKKAN DEY**, SHUVOJIT BANERJEE**

Cigarette smoking is the greatest single cause of various life-threatening diseases including cancer of the lung and other organs, chronic obstructive pulmonary disease, and cardiovascular disease. Smoking results in death or disability for half of all people who continue to smoke. Besides mortality and morbidity smoking results in loss of billions of dollars due to loss of productivity and health-care expenditure. The best and simplest way of prevention of smoke-induced diseases is to quit smoking. However, it has been unachievable because smokers are unable to kick the habit. We consider that an alternative way for prevention of cigarette smoke (CS)-induced diseases is to identify the disease-relevant chemical(s) in CS and inactivate it. We have observed that p-benzoquinone derived from cigarette smoke is a causative factor for smoke-induced pathogeneses. In this review we show that vitamin C, a strong antagonist of p-benzoquinone, prevents CS-induced various diseases.

A Few Facts about Cigarette Smoke

Smoking was the second leading risk factor for early death and disability worldwide in 2015. It has claimed more than 5 million lives every year since 1990 and its contribution to overall disease burden is growing, especially in lower income countries[1]. Despite more than half a century of unequivocal evidence of the harmful effects of tobacco on health in 2015, one in every four men in the world is a daily smoker.

Cigarette smoke (CS) contains about 4000 compounds: 3000 in the gas phase and 1000 in the tar phase[2]. Most of the disease producing chemicals are present in the tar phase that contains amongst others long-lived semiquinones including p-benzosemiquinone (p-BSQ)[3]. Smoke from commercial cigarettes contains substantial amounts of p-BSQ (100 - 200µg/cigarette)[4]. p-BSQ is not present in tobacco. It is formed during burning[5]. In the lungs, p-BSQ is converted to p-benzoquinone (p-BQ) by disproportionation and oxidation by transitional metal containing proteins, e.g. CU^{+2}-SOD, Fe^{+3}-cyt C[5, 6]. We have observed that p-BQ derived from CS is a causative factor for various CS-related diseases. In the lung, p-BQ causes emphysema, the most prominent pathological feature of COPD[5,6]. From the lungs, p-BQ gets into the blood where it forms covalent adducts with serum albumin as well as hemoglobin resulting in alteration of structure and function of the proteins[7,8]. Carried by the systemic circulation, p-BQ reaches the distant organs and causes various diseases. In the heart, p-BQ causes cardiovascular damage (CVD), including myocardial infarction and thrombosis[9]. In the bone marrow, p-BQ is a causative factor of myelodysplastic syndromes (MDS), a type of cancer[10]. In the kidney, p-BQ causes carcinoma in situ (CIS) of the renal pelvis[11]. We have observed that p-BQ also reaches the eye of smokers and is responsible for the formation of cigarette smoke-induced cataract. p-BQ is a redox cycling agent and it also forms covalent adducts with proteins (Michael adducts). In all the above-mentioned pathogeneses, p-BQ-induced oxidative damage and covalent adduct formation

* 1st Urmila and Mrinal Dewanjee Endowment Lecture delivered by Professor I. B. Chatterjee on 24th April, 2017.

** Department of Biotechnology and Dr. B. C. Guha Centre for Genetic Engineering and Biotechnology, Calcutta University College of Science, 35, Ballygunge Circular Road, Kolkata 700019.

are the initial events. Since vitamin C reduces and thereby inactivates p-BQ, we used vitamin C for the prevention of the CS-related aforesaid diseases.

Vitamin C: the Unique Antioxidant Vitamin against Oxidative Stress

The function of vitamin C as an antidote for counteracting oxidative stress is linked with the evolution of vitamin C synthesizing capacity in the terrestrial vertebrates. In most mammals, vitamin C is synthesized from glucose via the glucuronic pathway of metabolism: D-glucose → D-glucuronic acid → L-gulonic acid → L-gulonolactone → 2, keto-L-gulonolactone → L-ascorbic acid (vitamin C). L-gulonolactone oxidase (LGO, EC1.1.3.8) is the terminal enzyme in the pathway of biosynthesis of ascorbic acid in animals, which oxidizes L-gulonolactone to 2, keto-L-gulonolactone. 2, keto-L-gulonolactone is spontaneously converted to L-ascorbic acid (vitamin C)[12]. The expression of LGO took place in the early terrestrial vertebrates in the Devonian period (416 million to 358 million years ago), when the concentration of oxygen in water was 0.49% but the atmospheric oxygen concentration was 15–18%[13]. This would indicate that during evolution in the terrestrial atmosphere, the early vertebrates (amphibians) were exposed to an environmental oxygen concentration of 31–37 times that of their aquatic ancestors. This was a severe hyperoxic stress and an acute constraint for respiratory adaptation and survival of the early terrestrial vertebrates on land. Presumably, the emergence of LGO in the newly evolved vertebrates was apparently to provide the terrestrial vertebrates with adequate amount of ascorbic acid, a vital antioxidant, to protect their tissues against oxygen toxicity. In the evolutionary progress, the LGO gene became nonfunctional and the capacity to synthesize vitamin C was lost in the guinea pig, the primates, including humans[14,15,16,17,18]. The animals incapable of synthesizing ascorbic acid must consume enough vitamin C through their diet not only to prevent scurvy but also to survive against oxidative stress[19]. Since humans are incapable of synthesizing vitamin C and are totally dependent on the dietary vitamin C, the need of the vitamin for humans would be expected to increase several times when exposed to environmental oxidative stress such as cigarette smoking.

Pathophysiologies Caused by CS/p-BQ Exposure

Chronic Obstructive Pulmonary Disease (COPD): COPD is currently the fourth leading cause of death worldwide. In 2015, about 3 million deaths were caused by this disease and it was almost 5% of all deaths globally occurred in that year. It is predicted that COPD will cause 7.8 % of global death by 2030. Besides mortality, COPD attributes morbidity, productivity loss, and presents a great burden to both individual patients and the health care system. COPD is projected to be the 7th leading cause of *Disability-Adjusted Life Year (DALY)* in 2030. Unfortunately, none of the current therapies can alter the natural history of the disease and prolong survival. Since investigations on cigarette smoke-induced pathogenesis in human subjects are difficult, we used a guinea pig model for our studies. This is because guinea pig, like human is incapable of synthesizing vitamin C. Also guinea pig has anatomical and smoke-induced pathophysiological similarities to human[6,10]. We observed that after continuous exposure of guinea pigs to cigarette smoke or treating the guinea pigs with p-benzoquinone (intramuscular injection), in amounts approximately produced in the lung from CS exposure, there was progressive accumulation of p-benzoquinone in the lung. This was accompanied by emphysema, a phenotypic form of COPD. The mechanisms involved were arylation, oxidative stress, inflammation, apoptosis and proteolysis. Vitamin C (30mg/kg body weight/day) prevented accumulation of p-benzoquinone in the lung and the pathogenesis of emphysema. Our study provided the first proof that p-benzoquinone closely mimicked CS-induced emphysema and that a moderately high dose of vitamin C might be a simple preventive therapy for COPD in chronic smokers[6].

COPD is an insidious disease. At the initial stage, the diagnosis is often missed or delayed until the condition is advanced. When COPD is diagnosed, it is often too late to treat and cure. Cigarette smoking accounts for approximately 90% of COPD cases. There are more than one billion smokers in the world. However, it is not known which smoker will develop COPD in future. Generally, about 15-20% of smokers have COPD. At present there is no current method to identify asymptomatic smokers at risk for COPD. In a cross-sectional study with smokers with and without COPD, we observed that 90% of smokers with COPD had very low anti-p-BQ antibody in their serum. On the contrary, about 84% of smokers without COPD had high anti-p-BQ antibody. However, about 14% of smokers without COPD had practically little antibody. We considered that these 14% of the subpopulation might develop COPD in future. This clinical biomarker will facilitate development of novel drugs or other effective strategies for intervention of COPD at an early stage. Since

oral supplementation of vitamin C prevents CS/p-BQ-induced emphysema in the guinea pig, we consider that in addition to intensive smoking cessation efforts, intake of adequate vitamin C may prevent COPD in asymptomatic smokers having low anti-p-BQ antibody. However, our observations and propositions warrant further research for validation and translation in clinical practice[20].

Cardiovascular Disease (CVD) : Cardiovascular disease (CVD) including coronary heart disease and myocardial infarction (MI) is the leading global cause of death, accounting for more than 17 million deaths per year, a number that is expected to grow to more than 23 million by 2030. About one-third of CVD is caused by cigarette smoking. We produced myocardial injury in CS-exposed guinea pigs as evidenced by the release of cardiac Troponin-T and Troponin-I in the serum. These proteins are released when the heart muscle has been damaged. The mechanisms of CVD were oxidative stress, inflammation, apoptosis and thrombosis in the myocardium. We observed that p-BQ was a major factor responsible for CS-induced myocardial damage. Similar observations were made when equivalent amount of p-BQ produced from CS was injected to guinea pigs, thereby showing that p-BQ mimicked CS-induced myocardial injury. A moderately large dose of vitamin C (30 mg/kg/day) prevented CS/p-BQ-induced myocardial injury. The results indicate that dietary supplementation of vitamin C may be a novel and simple therapy for the prevention of pathological cardiovascular events in habitual smokers[9].

Myelodysplastic Syndromes : Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematological disease characterized by bone marrow hypercellularity, dysplasia, various degrees of cytopenia and a risk of progression to acute myeloid leukemia. Approximately one-third of patients ultimately progress to AML. MDS patients have a poor survival. The etiology of myelodysplastic syndromes (MDS) is largely unknown. Exposure to cigarette smoke (CS) is reported to be associated with MDS risk. We have observed that the causative agent of CS-induced MDS was p-BQ. Carried by the systemic circulation, p-BQ derived from CS reached the bone marrow, the target organ, and produced MDS. p-BQ is detoxified and thereby inactivated by NAD(P)H-quinone: oxidoreductase 1 (NQO1). There is evidence that deficiency of NQO1 increases the risk of MDS. Understanding the relationship among CS exposure, NQO1 deficiency in population-based studies is problematic. We used a guinea pig model for our study. We observed that three factors combined together led to CS-induced MDS: exposure to CS, NQO1 deficiency and marginal vitamin C deficiency. The initial event was oxidative stress as evidenced by the formation of protein carbonyls and 8-oxodeoxyguanosine. In recent years, the biomarker 8-oxodG has become pivotal for measuring the effect of endogenous oxidative damage to DNA and as a factor influencing the initiation and promotion of carcinogenesis. Oxidative damage was followed by apoptosis. Apoptosis disappeared later that was accompanied by hyperplasia and MDS. It is reported that cells that escape apoptosis survive to proliferate resulting in hypercellularity and MDS. Numerical chromosomal aberrations, aneuploidy, are common in cancer including hematopoietic tumor cells. We have observed aneuploidy in the MDS guinea pigs. The number of chromosomes in normal guinea pigs is 62. However, In CS-induced MDS guinea pigs, the number varied from 116 to 128. Supplementation of a moderately high dose of vitamin C (30 mg/kg body weight/day) prevented the myelodysplastic changes in CS-exposed NQO1-deficient guinea pigs. If the results obtained with guinea pigs are applicable to human, smokers having NQO1 deficiency conjoint with marginal vitamin C deficiency would be at high risk for developing MDS. Nevertheless, intake of a moderately high dose of vitamin C should prevent occurrence of MDS in smokers[10].

Carcinoma in Situ (CIS) of the Renal Pelvis : Carcinoma in situ (CIS) of the renal pelvis is an urothelial carcinoma (UC). UC is the fourth most common malignancy in men. It is a disease characterised by multiplicity, recurrence and multifocality. Urothelial CIS is a flat high grade non-invasive neoplasm, 60-80% of which becomes invasive in 5 years. We had mentioned above that p-BQ produced from p-BSQ goes to distant organs through systemic circulation. Here we show that p-BQ reaches the renal pelvis and causes CIS. We demonstrated before that p-BQ induces proliferation of cultured human lung cells through activation of epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) pathway. Persistent EGFR signaling often leads to deregulation of the Ras-MAPK pathway that ultimately leads to oncogenesis. MAPK pathway is directly coupled with cell cycle regulation. Here, using a guinea pig model we showed that prolonged treatment with p-BQ led to carcinoma in situ (CIS) of the renal pelvis. The mechanisms of carcinogenesis were p-BQ-induced oxidative damage and apoptosis that were later suppressed and followed by

activation of epidermal growth factor receptor, aberrant phosphorylation of intracellular tyrosine residues, activation of MAP kinase pathway and persistent growth signaling. This was accompanied by deregulation of cell cycle as shown by marked decrease in the expression of p21waf1/cip1 and cyclin D1 proteins as well as hyperphosphorylation of pRb. CIS has been characterised by histopathology and immunohistochemistry showing aberrant CK20, increased Ki- 67, and marked p53 nuclear immunopositivity with uniformly negative labelling of CD44. Oral supplementation of vitamin C (30 mg/kg body weight/day) prevented CIS of the renal pelvis and dysplasia in the ureter and bladder. Since majority of non-invasive UC progresses to invasive cancer with increased risk of mortality, our preclinical study might help to devise effective strategies for early intervention of the disease. We consider that our study would provide a mechanistic rationale for exploring the use of vitamin C for prevention of cancer of the renal pelvis (CRP) in smokers[11]. The dose of 30mg/kg body weight/day vitamin C in guinea pigs is roughly equivalent to 2g vitamin C/human smoker/day, which is the tolerable upper intake level of vitamin C in an adult human[21].

Conclusion

Majority of smokers are aware that habitual smoking usually leads to many life-threatening diseases. However, once addicted, it's tough to kick the habit of smoking. Quitting smoking is apparently out of a person's conscious control, particularly because of addiction to nicotine, a constituent of tobacco smoke that exerts its psychoactive effects. Although FDA-approved line 1st drugs are used for smoking cessation, only a limited number of smokers are successful at quitting. Prevention of smoke-related diseases is a global precedence. We consider that an alternative way of preventing smoke-related diseases is adequate intake of vitamin C (≈ 2g/day in divided doses). ❐

References

1. M.H. Forouzanfar, A. Afshin, L.T. Alexander, et al. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **388:** 1659–724 (2016)
2. B. W. Stewart and P. Kleihues (eds), World cancer report 2003 (Lyon, France: International Agency for Research on Cancer) p. 21–28 (2003)
3. K. Panda, R. Chattopadhyay, M. K. Ghosh, D. J. Chattopadhyay and I. B. Chatterjee, Cigarette smoke-induced protein oxidation and proteolysis is exclusively caused by its tar phase: prevention by vitamin C. *Toxicol. Lett.* **123:** 21–23 (2001)
4. N. Dey, A. Das, A. Ghosh and I. B. Chatterjee, Activated charcoal fi lter effectively reduces p-benzosemiquinone from the mainstream cigarette smoke and prevents emphysema. *J. Biosci.* **35:** 217–230 (2010)
5. S. Banerjee, *et al.*, R.Chattopadhyay, A. Ghosh, H. Koley, K. Panda, S. Roy, D. Chattopadhyay and I. B. Chatterjee, Cellular and molecular mechanisms of cigarette smoke-induced lung damage and prevention by vitamin C. Journal of Inflammation (London) **5:** p. 21 (2008)
6. A. Ghosh*, S. Ganguly*, N. Dey, *et al.* Causation of cigarette smoke–induced emphysema by p-benzoquinone and its prevention by vitamin C. Am. J. Respir. *Cell Mol. Biol.* **52** (3**):** p. 315–322 (2015)
7. A. Ghosh, A. Choudhury, A. Das, *et al.* Cigarette smoke induces p-benzoquinone–albumin adduct in blood serum: implications on structure and ligand binding properties. *Toxicology* **292** (2–3**):** p. 78–89 (2012)
8. A. Ghosh, S. Banerjee, A. Mitra, M. Muralidharan, B. Roy, R. Banerjee, A. Mandal and I. B. Chatterjee, Interaction of p-benzoquinone with hemoglobin in smoker's blood causes alteration of structure and loss of oxygen binding capacity. *Tox. Rep.* **3:** p. 295-305 (2016)
9. A. Das, N. Dey, A. Ghosh, S. Das, D. J. Chattopadhyay and I. B. Chatterjee, Molecular and Cellular Mechanisms of Cigarette Smoke-Induced Myocardial Injury: Prevention by Vitamin C. *PLoS ON.* **7:** e44151 (2012)
10. A. Das, N. Dey, A. Ghosh, T. Das, and I. B. Chatterjee, NAD(P)H: Quinone Oxidoreductase 1 Deficiency Conjoint with Marginal Vitamin C Deficiency Causes Cigarette Smoke Induced Myelodysplastic Syndromes. *PLoS ONE,* **6**: e20590 (2011)
11. S. Ganguly, A. Chandra, D. J. Chattopadhyay and I. B. Chatterjee, p-Benzoquinone initiates non-invasive urothelial cancer through aberrant tyrosine phosphorylation of EGFR, MAP kinase activation and cell cycle deregulation: Prevention by vitamin C. Tox. Rep. **4:** p. 296-305 (2017)
12. I. B. Chatterjee, Evolution and the biosynthesis of ascorbic acid. *Science* **182:** p. 1271–1272 (1973)
13. J. B. Graham, R. Dudley, N. M. Agullar and C. Gans, Implications of the late Palaeozoic oxygen pulse for physiology and evolution. *Nature* **375:** p. 117–120 (1995)
14. I. B. Chatterjee. Evolution and the biosynthesis of ascorbic acid. *Science* **182:** p. 1271–1272 (1973)
15. I. B. Chatterjee, N. C. Kar, N. C. Ghosh and B. C. Guha Biosynthesis of L-ascorbic acid: Missing steps in animals incapable of synthesizing the vitamin; *Nature* **192:** p. 163-164 (1961)
16. I. B. Chatterjee, H. A. Price and R. W. McKee. Biosynthesis of L-ascorbic acid in different subcellular fractions of prenatal and postnatal rat livers. *Nature* **207:** p. 1168-1170 (1965)
17. I. B. Chatterjee, J. J. Ghosh, N. C. Ghosh, G. C. Chatterjee and B. C. Guha, Identification of 2,keto-L-gulonolactone as an

intermediate in the biosynthesis of L-ascorbic acid. *Naturwissenchaften* **46:** p. 475-476 (1959)

18. M. Nishikimi, R. Fukuyama, S. Minoshima, N. Shimizu, K. Yagi, Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono lactone oxidase, the enzyme for Lascorbic acid biosynthesis missing in man. *J. Biol. Chem.* **269:** p. 13685–13688; (1994)

19. A. Nandi, C. Mukhopadhyay, M. K. Ghosh, D. J. Chattopadhyay, and I. B. Chatterjee, Evolutionary significance of ascorbic acid synthesizing capacity in terrestrial vertebrates. *Free Radical Biol. Med.* **22:** p. 1047-1054 (1997)

20. S. Banerjee, *et al.* P. Bhattacharyya, S. Mitra, S. Kundu, S. Panda, I. B. Chatterjee. Anti-*p*-benzoquinone antibody level as a prospective biomarker to identify smokers at risk for COPD. *Int. J. Copd.* **12:** p. 1847–1856 (2017)

21. Institute of Medicine. Food and Nutrition Board. Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. Washington, DC, National Academy Press, (2000)