SECTION-B

PROTECTIVE EFFECT OF ASCORBIC ACID AGAINST THE CLASTOGENIC XENOESTROGENS - TAMOXIFEN CITRATE AND METHYL PARATHION
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1. INTRODUCTION
1.1 Anticlastogenesis – History and Significance :

In the present industrial world, exposure to chemicals of various classes through different routes and means has become unavoidable. We have no choice but to be exposed to mutagenic and carcinogenic agents directly or indirectly. Although it has been recommended since many years to prohibit the genotoxic agents from the human environment by warning against their use and distributions, it is not achieved in general, due to many reasons. Absolute prohibition of mutagenic agents is not realistic in many fields (e.g. natural contamination of our food by pesticides; specific working places), or would even be detrimental in some cases (e.g. life-saving cancer therapies or immunosuppressive therapies). Therefore, finding out the possibilities of protecting the genetic material of exposed individuals from deleterious effects of environmental mutagens is essential (Odin, 1997).

Realizing the impact of chemicals on the ecosystem in general and man in particular and the concern that many innocuous chemicals could be highly genotoxic at concentrations that produced no other recognizable toxic effects, efforts are being made to find the agents capable of minimizing their genotoxicity (Gentile et al., 2001). There is an increasing evidence that cancer and other mutation-related diseases can be prevented not only by avoiding exposures to recognized risk factors but also by favouring the intake of protective factors and by modulating the defence mechanisms of the host organism. This strategy, referred to as chemoprevention, can be pursued either by means of suitable pharmacological agents and/or by dietary factors. Chemoprevention has become one of the most important research areas in the field of mutation and cancer research (Editorial, 2005).

The first step towards antimutagenic protection was initiated in the field of radiation biology when substances were detected which efficiently protected biological systems from the toxic effects of ionizing radiation. These substances, therefore, were called antimutagens, a term which, was first used by Novick and Szilard (1952) to describe the reduction of spontaneous or chemically induced mutagenic damage in bacteria. Since the action of ionizing radiations and certain chemicals on the genetic material is very similar, it was consequently presumed that antimutagens should also reduce the damaging activity of those chemicals on the genetic material. This assumption was experimentally proved in the 1950s in bacteria.
Despite the technical problems hampering the progress in antimutagen studies during those days, extensive reviews on antimutagens were published (Gebhart, 1974; Clarke and Shankel, 1975). Antimutagen research experienced a renaissance in the 1980s when the interest of researchers increasingly focused on natural antimutagens. Since the report of Novick and Szilard (1952), many of the short-term tests for genetic toxicity also have been used to identify antimutagens. By extension, it appears to be widely assumed that these tests can also be used to identify potential anticarcinogens (Brockman et al., 1992). The main goal of antimutagenicity research is to protect the human gene pool from the harmful effects of environmental chemicals with a view to reduce the risk of hereditary diseases and cancers (Gebhart and Arutyunyan, 1991). Anticlastogenesis studies on plant cell material date back to the 1950s (Avanzi, 1957). Also a large series of studies have been performed on human cell material, mainly cultures of peripheral blood lymphocytes in the later 1960s. These investigations not only elucidated many of the mechanisms governing anticlastogenic action, but also led to methodological details and approaches for testing anticlastogenicity (Gebhart, 1992).

Anticlastogens are agents, which can reduce the amount of chromosomal damage induced by clastogenic agents (Klaunig and Kamendulis, 2004). The idea of reducing clastogenic action by anticlastogens achieved an additional extraordinary topicality by some of the findings of modern tumor cytogenetics and oncogenetics, which unequivocally have demonstrated the intimate involvement of mutational changes, particularly chromosomal rearrangements and deletions, in the multistep process of malignant transformation and cancer development (Heim et al., 1987a&b; Kristofferson et al., 1987; Mandahl et al., 1987; Lundgren et al., 1992; Akervall et al., 1995; Schrock et al., 2006; Senthamizhchelvan et al., 2006; Ol’shanskaia et al., 2006). As a consequence, a reduction in the frequency of induced chromosomal damage, beside the well-known genetic risks, should also diminish the cancer risk in a clastogen-exposed population. With the growing evidence of an extremely close relation between mutagenesis and carcinogenesis, the aspect of protection was extended to anticarcinogens (De Flora, 1988).
During the past few years, antimutation and anticancer research has continued to grow steadily. Many special conferences and congresses entirely devoted to this topic have addressed the various issues related to this aspect. A growing proportion of the international literature in the general field of mutagenesis and carcinogenesis is nowadays devoted to the issues of inhibition and modulation (De Flora et al., 1992). Since last few decades, a large number of protective compounds have been identified which are christened as antimutagens, anticarcinogens and anticlastogens.

Thus, in brief, anticlastogenesis is an essential part of antimitagenesis, and perhaps also of anticarcinogenesis. It obtained its justification and its character as an independent field of research from the mutational endpoints, which are the subject of anticlastogenesis studies. A considerable amount of the data obtained in this field has been generated through experimental studies in mammalian system and human cell cultures. Basic and applied research on anticlastogenesis has not only revealed valuable evidence of the mechanism active in the induction of chromosomal aberrations by environmental mutagens and of the principles of their modulation, but also contributed effective ideas on the practical application of this knowledge for the protection of individuals at risk (Gebhart and Arutyunyan, 1991). Weighty stimuli for investigation in this field have also come from the exploration of the process of malignant transformation. Several experimental evidences have shown that cancer is ultimately generated by mutational processes in somatic cells, and chromosome anomalies form a considerable part of them. Thus, apart from the genetic protection of future generations against the detrimental effects of mutagens on germ cells, evidence is growing that somatic cells of exposed individuals must also be protected from genotoxic actions.

At present, an extensive research is underway in this field and several natural compounds, plant extracts, dietary components and antioxidant vitamins have been studied for their antimutagenic and anticlastogenic actions against wide variety of chemicals and radiation (Sasaki et al., 1990; Chorvatovicova and Sandula, 1995; Edenharder et al., 1998a&b; Xue et al., 1998; Antunes et al., 1999; Rao et al., 2001; de Lima et al., 2001; Prasad et al., 2002; Fahmy et al., 2002; Guha and Khuda-Bukhsh, 2003; Mikulasova and Kosikova, 2003; Negraes et al., 2004; Selvakumar et al., 2006).
Compounds gathered under the common term vitamins are necessary for living organisms due to numerous important biological functions performed by them. Xenobiotics that induce mutation and cancer undergo metabolic changes and during this process reactive free radicals such as oxygen radicals, hydrogen peroxide, superoxide etc. are generated. Oxygen derived free radicals are capable of severely damaging cells by oxidizing macromolecules such as proteins and DNA (Barber and Harris, 1994). The role of estrogens and xenoestrogens in the generation of reactive oxygen species leading to oxidative stress and damage in the genetic material is well documented (Anderson et al., 2003). Many natural and synthetic chemicals with antioxidant properties have been shown to scavenge the free radicals and thereby protect the cells from mutagenic effects. Vitamins are very well known free radical scavenger. Three vitamins, namely, E, A and C, are thought to be the main protective agents in this class of compounds (Odin, 1997). These three substances have been extensively studied in the last two to three decades and many beneficial effects have been found following the tests on bacterial, plant and mammalian cells. They were shown to inhibit the genotoxic action of many chemical mutagens, radiation and virus-induced cell transformation. It is possible that these vitamins may be included in the defense systems of organism and protects cells and tissues against harmful exogenous and endogenous factors (Akturk et al., 2006).

Because of the relationship between mutagenicity and carcinogenicity, it can be assumed that increased levels of natural antimutagens will be beneficial in preventing induction of cancer (Odin, 1997). In various experimental results and epidemiological studies, it has been revealed that vitamins provide examples of both inhibition and modulation of tumor growth treated with anticancer drugs/therapy (Wattenberg, 1981; Newberne and Suphakarn, 1984; http://darwin.nap.edu/openbook.php?record_id=371&page=138). Antioxidant vitamins have been extensively studied recently for their capacity to protect human cells from damage induced by radiation and chemicals. Their protective effect on chromosomal damage has also been proved in rodent bone marrow cells (Odagiri et al., 1992; Sarma et al., 1993; Abraham et al., 1993; Salvadori et al., 1996; Konopacka et al., 1998; Konopacka and Rzeszowska-Wolny, 2001).
1.3. Anticlastogenic effects of ascorbic acid (AA; Vitamin-C):

Since its discovery about 75 years ago, vitamin-C (VC) is known to be a 'wonder worker' in the biological systems. In addition to its role in collagen formation and other life-sustaining functions, VC serves as a key immune system nutrient and a potent free-radical scavenger. This double-duty nutrient has been shown to prevent many illnesses from everyday ailments such as the common cold to devastating diseases such as cancer (Null, 1994). In the scientific world, the water-soluble VC is known as ascorbic acid (AA) (meaning "without scurvy", the disease caused by VC deficiency). Even though, humans depend on ascorbic acid for many aspects of biochemical functioning; yet are among only a handful of animal species, who cannot produce their own supply of AA. Therefore, they have to depend on the supply of AA from external sources. Whether derived from exo or endogenous source, AA, the enolic form of 3-keto-L-glucofuranolactone is among the most common metabolites of living organisms. It takes an active part in tissue metabolism and is connected with numerous electron-transport processes, where it behaves as a strong reducing agent. The same reducing property makes AA an excellent antioxidant, capable of scavenging a wide variety of different oxidants/free radicals, which are responsible for inducing the mutation. (Uddin and Ahmad, 1995).

Well over a thousands of studies were carried out and reports were published on the protective effect of AA against various types of mutagenic compounds in a wide spectrum of test systems both in vitro and in vivo. Yoshikawa et al. (2006) elucidated the protective effect of AA against double-strand breaks in reconstituted chromatin visualized by single-molecule observation. Egel et al. (2002) studied the inhibitory effects of AA against the pyrimethamine induced chromosomal aberrations in vitro. It has been demonstrated that supplementation of AA decreased the endogenous level of oxidative damage of DNA in lymphocytes and increased the resistance to damage induced in vitro by ionizing radiation and chemicals (Anderson et al., 1997; Duthie et al., 1996; Noroozi et al., 1998; Konopacka and Rzeszowska-Wolny, 2001). Antimutagenicity of vitamin C against mutagenic complex mixture has been elucidated in S. typhimurium strain TA98 (Ong et al., 1989). Gentile et al. (1998) showed antigenotoxic property of certain antioxidants including AA against
the induced genotoxicity of anticancer drugs such as Bleomycin, Cisplatin, Cyclophosphamide, Doxorubicin using *Salmonella* microsome bioassay. Kaya et al. (2002) demonstrated the modulatory effect of ascorbic acid on genotoxicity using the wing spot test in *Drosophila*. There are many reports on the protective effects of AA in animal bone marrow cells. AA decreased the frequency of micronucleated cells induced by the antiamoebic drug diiodohydroxyquinoline in bone marrow of Swiss albino mice (Ghaskadbi and Vaidya, 1989). Peroral supplementation with AA readily decreased the number of cells with micronuclei induced by Cr(VI) in rat and guinea pig bone marrow (Ginter et al., 1989). Konopacka and Rzeszowska-Wolny (2001) worked out the protective effect of VC along with VE and B-carotene against the genetic damage induced before and after gamma-ray irradiation in human lymphocytes *in vitro*. AA induced anticlastogenic effects against the therapeutic/antitumour agents have been demonstrated in several *in vitro* and *in vivo* studies (Kola et al., 1989; Giri et al., 1998; Vijayalaxmi and Venu, 1999; Antunes et al., 2000; Aly and Donya, 2002) There are several reports on protective effects of AA against the clastogenicity of pesticides (Joseph and Usharani, 1993; Hoda and Sinha, 1993; Crott and Fenech, 1999; Siddique and Afzal, 2005). AA reduced the aluminum induced sister-chromatid exchanges in mouse bone marrow cells (Dhir et al., 1993). There are also many experimental evidences in support of the protective effect of AA performed on human groups exposed occupationally to increased doses of chemical mutagens. Sram et al. (1983) showed the protective effect of ascorbic acid prophylaxis against the risk of genetic injury (chromosome aberrations) in a group of coal-tar workers. Similar tests established the antimutagenicity of AA, which diminished the level of aberrations in cancer patients who have been treated with Bleomycin (Pohl and Reidy, 1989). It has also been reported that dietary AA protects human sperm from endogenous oxidative DNA damage that could affect sperm quality (Fraga et al., 1991). Thus, there are ample epidemiological and experimental evidences in support of genoprotective effects of AA against a wide variety of agents. In the words of Odin (1997), "Experiments of this type not only have theoretical significance, but also have a high practical value by demonstrating AA as a possible therapeutic remedy for prevention of genetic damage".

Considering all these positive aspects of AA and its non-toxicity in *in vivo* system even at high doses, AA was selected in the present anticlastogenesis study to
elucidate its possible protective role against the clastogenesis induced by two xenoestrogenic compounds, one representing the therapeutic drugs (Tamoxifen citrate) and the other organophosphorous pesticides (Methyl parathion).

1.4. **In vivo cytogenetic endpoints for anticlastogenesis study:**

The elucidation of modulatory effect of desmutagens or bioantimutagens in bacteria, which has become a fashion for the fast few years, does not answer the question of the practical feasibility and efficiency of antimutagens in humans. Therefore, definite intensification of *in vivo* research on antimutagens particularly anticlastogen research by making use of suitable systems is crucially needed. Mammalian assays continue to be useful to explore both mechanism and effects of antimutagens (Lohman et al., 2001). A lot of *in vitro* studies in human lymphocyte culture and various other cell cultures were carried out to evaluate the anticlastogenesis of various antioxidants. However, *in vitro* study has got its own limitations. An established antigenotoxic response in *in vitro* test systems should be verified in *in vivo* test systems (Ferguson, 1994), taking into account the limitations of target cell concentrations and exposure of target cells. Therefore, if antigenotoxic potential in *in vivo* test system is not evident, then consideration of that agent as an antigenotoxic substance is not appropriate (Gebhart and Arutyunyan, 1991). A series of mammalian *in vivo* test systems have become indispensable tool in modern genetic toxicology because of their metabolic similarities to humans, their ability to detect a variety of genetic damage, and their high reliability. Therefore, these systems are also utilized extensively in anticlastogen research. The endpoint, which directly reflects chromosomal changes, is also most suitable to elucidate the practically relevant and efficient anticlastogenic action. In fact, substances that reduce the amount of chromosomal damage induced by clastogens are termed as ‘anticlastogens’ (Gebhart and Arutyunyan, 1991). Considering the immense practical impact of chromosomal anomalies on human health in terms of chromosomal disorders and tumor cytogenetics, the data obtained from these studies gains direct practical importance. The protection of chromosomes from the damaging activity of known mutagens has been under study since 1950s using well-defined chromosomal aberration test (Ito et al., 1986; Mukherjee et al., 1991; Antunes et al., 2000; Prasad et al., 2002; Samarth
The MN assay is among the most well validated endpoints for use in chemoprevention trials. There are many advantages in using this assay, i.e., MN represents a change, which is biologically relevant to the process of carcinogenesis. Any change in MN frequency in presence of clastogen alone and in combination with protective agent can clearly be quantified, and in many cases the assay can be run noninvasively. Moreover, the MN assay is easier to apply and it is quite reasonable to apply this method on a large scale in risk groups exposed to genotoxic agents and to study the effects of anticlastogens. Antioxidant/anticlastogenic property of most of bioantimutagens were carried out employing the chromosomal aberration analysis and MN assay, which throw light on the significance of these two parameters in anticlastogenesis study. Employing bone marrow MN assay, several studies have been carried out for the elucidation of anticlastogenicity against wide variety of genotoxic agents (Ghaskabdi et al., 1987; Ghaskadbi et al., 1992; Ghaskabdi and Vaidya, 1991; Chorvatovicova and Navarova, 1992; Abraham et al.; 1993; Vijayalaxmi and Venu, 1999; Delmanto et al., 2001; Goncharova et al., 2001; Jgetia and Reddy, 2002; Prasad et al., 2002; Premkumar et al., 2003).

Thus, considering the magnitude of in vivo anticlastogenicity investigation over that of in vitro parameters and relevance of CA test and MN assay in anticlastogenesis study, in the present investigation, the possible protective effect of AA was studied against the clastogenicity of two xenoestrogenic compounds viz, tamoxifen citrate and methyl parathion by employing MN assay and CA test in mouse test system.

1.5. Ascorbic acid -chemical nature, doses and treatment schedule:

L- Ascorbic acid (AA ,CAS no. 50-81-7, Lot no. 4719015 927 and product no. 40063N) purchased from BDH laboratory Supplies, England, was used for the experiment.

Molecular weight : 176.1
Melting point : 190°
AA is a water-soluble vitamin, available as odorless, colorless crystals or pale yellow crystalline powder with an acidic taste. It may be extracted from citrus fruits or may be prepared synthetically. Besides water, AA is soluble in alcohol and acetone. AA is readily absorbed from the gastro-intestinal tract and is widely distributed in the body tissues. Ascorbic acid in excess of the body's needs is rapidly eliminated in the urine and its elimination is usually accompanied by a mild diuresis. In its metabolism, AA is first converted into dihydroascorbate by a number of enzymes or nonenzymatic processes and then reduced in cells. In the present investigation, to assess the protective effect of AA on the genotoxicity of xenoestrogens under study, 3 doses were selected namely, 50, 100 and 200 mg/kg b.w., which fall within the dose range used in humans. The therapeutic dose for wound healing in adults is 2-3 gms, daily equivalent of 50mg/kg b.w and in the case of scurvy the dose would be between 200 to 500 mg daily. The minimum protective dose of AA against clinical scurvy is 10 mg daily for children, and for adults ranges between 30 - 60 mg daily (Martindale, 2002). On the basis of this information, we selected 3 different doses i.e., 50, 100 and 200 mg/kg b. w. of L-ascorbic acid (AA) in the present study.

For anticlastogenic studies, two protocols were followed, repeat-dose treatment of test chemical and cumulative dose treatment of AA. In the former case, one dose of AA was administered 24 hours before the administration of test chemical and second was given simultaneously with the test chemicals. In the cumulative treatment schedule, the test agents and ascorbic acid were administered simultaneously for five consecutive days. The test chemicals were administered orally in view of their principal route of exposure. AA is readily absorbed from the gastro-intestinal tract and widely distributed in the tissues (Martindale, 2002). Hence, oral
route of administration was chosen for AA also. Appropriate AA control groups were
maintained.