CHAPTER 2: REVIEW OF LITERATURE

2.1 Asthma

Asthma is the most chronic disease in children in developed nations (Palmer and Cookson, 2000). Asthma is a chronic pulmonary disorder with hyperresponsiveness of trachea and bronchi to a variety of stimuli (Aron et al., 1999; Lee et al., 2000; McFadden, 2005), variable obstruction in the airflow usually associated with inflammation (Los et al., 1999), and increased infiltration of inflammatory cells especially eosinophils in the airways (Xin et al., 2001).

Asthma is associated with chronic morbidity and mortality worldwide with around 300 million affected worldwide and over 180,000 deaths each year (Global Asthma Report, 2014). The prevalence is highest in the developed countries such as, Australia, New Zealand, United States, United Kingdom and North-west Europe (Global Asthma Report, 2014). Around half of the people with asthma develop it before age 10, and most before the age of 30. The frequency of asthma is twice as more common in boys than in girls, in young ages but, in adults the case is just reverse. Prevalence is higher in urban areas than rural. In 2012, asthma along with other respiratory diseases was reported to cause 4 million deaths globally (WHO, 2015).

Asthma is controlled by both genetic as well as environmental factors and both when combined exacerbate the disease. Asthma is a complex heritable disease and has a strong genetic component (Sibbald et al., 1980). The inheritance doesn’t show classical Mendelian pattern of inheritance (Los et al., 1999). Asthma is controlled by multiple interacting genes of which some are protective while others contribute to the disease pathogenesis which are influenced by the environment. There are more than 100 loci (both autosomal as well as X-linked) that are associated with asthma (Haagerup et al., 2002; Liu et al., 2004; Vercelli, 2008). To understand the complete etiology of asthma, it is important to study both genetic components and environmental factors that trigger asthma.

To identify the genes associated with asthma and associated phenotypes, a genome-wide screen was conducted by Ober et al. (1998) using 292 autosomal and 3
X-Y pseudoautosomal markers. 12 markers were identified in 10 regions that showed association with asthma and related phenotypes with possible linkage markers in four regions: 5q23-31, 12q15-24.1, 19q13 and 21q21.

Since the 1980s, numerous large studies have identified significant relations between parental smoking and development of asthma in children (Cook and Strachan, 1997, 1998; Jaakkola, 2000; Jaakkola and Jaakkola, 2002). Meta-analyses based on these studies have shown a dose-dependent increase in children’s rates of asthma related to increasing number of household smokers (Cook and Strachan, 1997), with the strongest effect detected in the youngest children (California EPA, 1997; Strachan and Cook, 1997, 1998a, b). Phase II detoxification enzymes, particularly classes of GSTs and NQO1 have been shown to play an important role in regulating inflammatory responses triggered by xenobiotic and oxidatively labile compounds (Cross et al., 1999; Baulig et al., 2003; Ahn et al., 2006; Wan and Diaz-Sanchez, 2006). The importance of genetic susceptibility in determining the risk of developing asthma from early childhood exposure was suggested by analyzing parental history of asthma and allergies as an index of genetic risk. In a study of California school children, it was found that having a mother who smoked during pregnancy was most strongly related to early-onset of persistent asthma among children with a parent with asthma or allergies (London et al., 2001).

Dietary factors appear to influence effects of air pollution on asthma-related phenotypes. Antioxidant intake was shown to reduce the effects of daily variation in ozone exposure on changes in pulmonary function in children with asthma in Mexico City (Romieu et al., 2002).

The morbidity and mortality due to asthma is higher among asthmatic smokers as compared to nonsmoking asthmatics. Also, smoking has a remarkable detrimental effect on pulmonary function in asthmatic subjects and it increases the risk of severe asthma exacerbation. Cigarette smoke also influences the efficiency of inhaled corticosteroid treatment in asthma. The association between environmental tobacco smoke exposure and decline in pulmonary function is well documented (Jaakkola and Jaakkola, 2002, 2006; Larsson et al., 2003; Eisner et al., 2005; Alipour et al., 2006).

Gilliland et al. (2002) reported effects of Glutathione S-transferase M1, maternal smoking during pregnancy, and environmental tobacco smoke on asthma and wheezing in children. Glutathione S-transferase (GST) M1 may play a role in asthma and wheezing occurrence among those exposed to tobacco smoke, as it
functions in pathways involved in asthma pathogenesis such as xenobiotic metabolism and antioxidant defenses. The effects of *in utero* exposure to maternal smoking on asthma and wheezing occurrence were largely restricted to children with GSTM1 null genotype.

Genetic variants can modulate the effects of prenatal tobacco smoke exposure on DNA methylation patterns. For example, risk of childhood asthma associated with prenatal tobacco smoke exposure differs by GSTM1 genotype. Children carrying the common null genotype whose mothers smoked during pregnancy are at greater risk for developing asthma (Gilliland *et al.*, 2002, 2004; Kabesch *et al.*, 2004). Children who are null for GSTM1 and exposed to second hand smoke (SHS) have a reduced peak expiratory flow rate (Palmer *et al.*, 2006). Haplotypes in GSTP1 and maternal smoking jointly increase the odds of childhood asthma (Li *et al.*, 2008).

### 2.2 Asthma and oxidative stress

Oxidative stress occurs when there is disturbance in the oxidant-antioxidant balance in the body in favor of the oxidants, leading to potential damage (Sies, 1991; Halliwell and Gutteridge, 2007). Reactive oxygen species are the oxygen radicals such as superoxide, singlet oxygen, hydroxyl, peroxy, hydroperoxyl, alkoxyl, and carbonate radicals and the non-radical derivatives of oxygen such as hydrogen peroxide, peroxynitrite, peroxynitrous acid and hypochlrous acid (Halliwell and Gutteridge, 1995, 2007). These are generated endogenously either by metabolic reactions from mitochondrial electron transport during respiration, or during activation of circulating inflammatory cells or phagocytes, as well as exogenously from air pollutants or cigarette smoke. There are two sources of ROS in the lungs, environmental and cellular. Environmental sources consist of both gaseous and particulate air pollution ranging from cigarette smoke and oxidant gases, such as ozone, nitrogen dioxide, and sulphur dioxide, to airborne particulate matter like vehicles’ exhaust fumes that can stimulate ROS production.

In response to an allergen, the acquired immune system is activated which is characterized by production of interleukin IL-5 and simultaneous activation and recruitment of eosinophils to the airways. The innate immune system on the other hand, act via production of IL-8 and subsequent activation and recruitment of neutrophils. This stimulated activation of immune cells in response to any allergen is called as “respiratory burst”. This involves uptake of O$_2$ and release oxygen radical
O$_2^-$ by a NADPH-dependent superoxide generating system which is then converted to hydrogen peroxide under the influence of superoxide dismutase (SOD). This can result in the non-enzymatic production of the more damaging OH$^-$ from H$_2$O$_2$ in the presence of ferrous (Fe$^{2+}$) through the Fenton reaction (Figure 2.1). This leads to the oxidation of ferrous (Fe$^{2+}$) to ferric (Fe$^{3+}$), which in turn can also generate OH$^-$ directly from superoxide radical and regenerate Fe$^{2+}$ through the Haber–Weiss reaction (Halliwell and Gutteridge, 1995, 2007, Wood et al., 2003). Redox cycling of Fe$^{2+}$ and Fe$^{3+}$ can therefore result in the formation of the more damaging OH$^-$. There are several other enzymes in macrophages to produce ROS such as, heme peroxidases, myeloperoxidase, or eosinophil peroxidase (EPO), which are also found in neutrophils and eosinophils, respectively. These enzymes catalyze the formation of the potent and very damaging oxidants hypochlorous acid (HOCl) and hypobromous acid (HOBr) from H$_2$O$_2$ in the presence of chloride and bromide ions, respectively (Wood et al., 2003; Genestra, 2007).

The reactive oxygen species are generally produced in the body but are scavenged by the antioxidant enzymes simultaneously. But, when a person is suffering from a degenerative disease, the over burst of inflammatory cells causes increased production of reactive oxygen species (ROS) that leads to severe damage to the cell. Cellular-derived ROS are produced by inflammatory and epithelial cells within the lungs. The sources for cellular-derived ROS include mitochondrial respiration, NADPH oxidase and xanthine/xanthine oxidase system (Laumbach and Kipen, 2010).

The ROS trigger specific signaling pathways, creating toxic products, alter gene expression and enzyme activity and disrupt normal repair mechanism (Poulson et al., 1998). ROS generate a large number of oxidative modifications in DNA, including strand breaks, base oxidations and formation of DNA adducts (Dizdaroglu et al., 2002; Cadet et al., 2003; Bjelland and Seeberg, 2003). These damage biomolecules, cell components, attack membrane lipids, proteins as well as nucleic acids causing lipid peroxidation, protein modification and DNA damage (Halliwell and Gutteridge, 2007).
Figure 2.1: Mechanism leading to lipid peroxidation in asthma (Wood et al., 2003). IL: interleukin; Th2: T-helper type-2 cells; NO₂: nitrogen dioxide; ROS: reactive oxygen species; O₂⁻: superoxide; H₂O₂: hydrogen peroxide; OH⁻: hydroxyl radical; NADPH: reduced nicotinamide-adenine dinucleotide phosphate; NADP: nicotinamide-adenine dinucleotide phosphate; SOD: superoxide dismutase; EPO: eosinophil peroxidase; MPO: myeloperoxidase; NF: nuclear transcription factor.
A variety of pathological respiratory diseases like asthma, allergic rhinitis, allergic bronchitis and chronic obstructive pulmonary disease, cardiovascular diseases, chronic diseases, such as atherosclerosis, diabetes, and rheumatoid arthritis, hypertension as well as other degenerative diseases like cancer, ageing and age-related diseases and neurodegenerative diseases are associated with oxidative stress (Rahman et al., 1996; Dhalla et al., 2000; Bowler and Crapo, 2002; Volkovavà et al., 2006; Khansari et al., 2009).

Chung (1986) reported increased ROS production during acute exacerbations in asthmatic subjects. There was a twofold increase in generation of ROS from neutrophils and fourfold from macrophages in asthmatic subjects in comparison with control subjects. There is respiratory burst of alveolar macrophages in BAL in asthmatic subjects, and a significant relationship between ROS generation by alveolar macrophages and severity of asthma (Cluzel et al., 1987). Macrophage ROS generation was associated with increased airway hyperresponsiveness (Kelly et al., 1988). The inflammatory cells that are recruited to the airways have a capacity of producing oxidants. Once recruited, these become activated and generate reactive oxidants in the airways, in response to various stimuli. Activated eosinophils, neutrophils, monocytes, and macrophages, and other resident cells such as bronchial epithelial cells, can generate oxidants (Barnes, 1998; Henricks and Nijcamp, 2001).

Higher concentration of hydrogen peroxide (Horvath et al., 1998; Emelyanov et al., 2001), superoxide anion radical (Sedgwick et al., 1990; Jarjour and Calhoun, 1994; Teramoto et al., 1996), nitric oxide (Ashutosh, 2000; Banovcin et al., 2009) has been reported in asthmatic patients. Increased formation of 3-bromotyrosine on proteins in the BAL of patients with asthma has been observed (Wu et al., 2000).

The level of oxidative stress is increased in the lungs as well as in the circulation of children and adults with asthma as suggested by many earlier studies (Kanazawa et al., 1991; Jarjour et al., 1992; Jarjour and Calhoun, 1994). The association between airway obstruction/severity of the disease and systemic oxidative stress suggests that blood has a central role because it transports and redistributes antioxidants to every part of the body (Davies, 1995; Evans and Halliwell, 2001). An imbalance between oxidants and antioxidants is proposed in smokers and in patients with airways diseases. Rahman et al. (1996) measured the Trolox equivalent antioxidant capacity (TEAC) of plasma and the levels of products of lipid peroxidation as indices of overall oxidative stress. The plasma TEAC was markedly
reduced with increased levels of lipid peroxidation products, in healthy chronic smokers as compared with healthy nonsmokers. Plasma TEAC was also low in patients presenting with acute exacerbations of chronic obstructive pulmonary disease (COPD) and asthma with increase in plasma lipid peroxidation products. Therefore smoking, acute exacerbations of COPD and asthma are associated with a marked oxidant/antioxidant imbalance in the blood, associated with increased incidences of oxidative stress. There are several factors that can trigger ROS production in the airways of asthmatic patients such as lipid mediators, chemokines, adhesion molecules, and eosinophil granule proteins. Through ROS generation, these trigger an increased inflammatory response generating many of the pathophysiological features of asthma, including airway smooth muscle contraction, hyperreactivity and increased secretions, increased vascular permeability, and increased synthesis of chemoattractants (Henricks and Nijkamp, 2001). 

Oxidative stress is a well known component as well as consequence of asthma pathogenesis (Bowler and Crapo, 2002; Wood et al., 2003; Bowler, 2004; Caramori and Papi, 2004) and there are evidences that the asthma and other allergic diseases are mediated by oxidative stress (Bowler and Crapo, 2002). The inflammation in the airways of asthma patients is due to increased vascular permeability, swelling of the airway walls, excessive secretion of mucus and infiltration of inflammatory and immune cells especially, eosinophils (Wood et al., 2003) which produce reactive oxygen species and other mediators (Andreadis et al., 2003). 

ROS interact with a wide variety of molecules in the biological systems resulting in lipid peroxidation and enzyme dysfunction and enhancement of proinflammatory cell signaling, thereby profoundly altering cellular function within inflammatory lung diseases (Poli et al. 2004). ROS causes contraction of airway smooth muscles which is enhanced when the epithelium is injured or removed. The injury caused by the reactive oxygen species to the airway epithelium produces hyperresponsiveness of the airways, suggesting that ROS play a role in the pathogenesis of asthma (Valko et al., 2007). Eosinophils play a crucial role in the inflammatory response in asthma, as they are present excessively in bronchoalveolar lavage (BAL) and blood that is associated with bronchial hyperresponsiveness. In vivo activation of eosinophils results in EPO release and oxidative damage to proteins through bromination of tyrosine residues (Valko et al., 2007).
The airways, lungs and blood have a well equipped antioxidant defense mechanism which protects against increased oxidants (Caramori and Papi, 2004) but, a decline in this defense system occurs during allergic diseases such as asthma and allergy with simultaneous increase in the oxidants because of inflammation, and leads to oxidative stress (Fujisawa, 2005; Sackesen et al., 2008). This is supported by the studies that have shown that inflammatory cells from peripheral blood and broncho alveolar lavage (BAL) fluid of asthmatic subjects generate more superoxide anion radicals than controls (Cluzel et al., 1987), as well as the exhaled air of patients with asthma contains elevated levels of various markers of oxidative stress (Baraldi et al., 2003; Corradi et al., 2003).

Ercan et al. (2006) determined the factors associated with oxidative stress including asthma severity and the genotype of the antioxidant enzymes. Children were genotyped for null variants of glutathione S transferase (GST) T1 and GSTM1, and ile105val variant of GSTP1 indicating the presence of a strong oxidative stress in children with asthma that increases with the severity of the disease and Children with asthma with val/val genotype at GSTP1 ile105val locus may be good candidates for supplemental antioxidant therapy.

It has been suggested by various dietary studies that intake of various foods that have antioxidant effects like fresh fruits, juices, vitamin C, vitamin E, vitamin A, retinols, α- and β- carotene affects the lung function and severity of asthma (Harik-Khan et al., 2004; Misso et al., 2005; Riccioni et al., 2007; Prescott and Dunstan, 2007). The increased production of ROS in asthma leads to alteration in key enzymatic as well as non-enzymatic antioxidants such as glutathione, vitamins C and E, beta-carotene, thioredoxin, superoxide dismutase, catalase, and glutathione peroxidase causing oxidant-antioxidant imbalance in airways which further leads to chronic inflammation (Nadeem et al., 2008). Zeyrek et al. (2009) reported that the oxidant-antioxidant balance is impaired in children with asthma bronchiale, which further leads to DNA damage. They observed that plasma oxidant and peroxide concentration was increased while total antioxidant status (TAS) of plasma was reduced in the patients as compared to controls and a positive correlation between plasma TAS and DNA damage was reported.
2.2.1 Antioxidant status and asthma

There is a well established antioxidant defense mechanism comprising both enzymatic and non-enzymatic antioxidants that combat and neutralize the deleterious effects of oxidative forces. The antioxidants are the molecules that are capable of stabilizing or deactivating the free radicals and prevent them from attacking the cells.

The oxidative stress in asthma is associated with alteration in the antioxidant activity in the lung and blood (Heffner and Repine, 1989; Sackesen et al., 2008). There are evidences that there is decline in total antioxidant capacity as well as individual antioxidants in plasma and BAL fluid of asthmatic subjects (De Raeve et al., 1997; Comhair et al., 2000; Kanazawa et al., 2002). Decreased antioxidant level in plasma and BAL fluid during stable asthma and in exacerbation, provides strong evidence for increased oxidative stress in asthma (Comhair et al., 2000). Antioxidants are present endogenously but these can also be obtained exogenously through diet or supplements. An ideal antioxidant should be readily absorbed, able to quench free radicals, chelate redox metals and work in both aqueous and membrane domains (Rahman, 2007). Patients with uncontrolled asthma show low level of plasma antioxidant level as compared to those with controlled or partly controlled asthma (Wood and Gibson, 2009).

There are various reports suggesting alteration in different antioxidants in asthma. However, the data is inconsistent, which could be due to variation in diet, body fluids or the technique used for analysis. Many studies have reported vitamin C deficiency in plasma/serum (Aderele et al., 1985; Vural and Uzun, 2000; Kalayci et al., 2000b), BAL fluid (Kelly et al., 1999) and whole blood (Shanmugasundaram et al., 2001), vitamin E deficiency in BAL fluid (Kelly et al., 1999), bronchial wash (Kelly et al., 1999), erythrocytes (Mohan and Das, 1997) and plasma/serum (Kalayci et al., 2000a; Shanmugasundaram et al., 2001). Whereas, some studies have reported a high level of vitamin E (Kelly et al., 1999) or no difference (Wood et al., 2000) in plasma/serum. Sackesen et al. (2008) have also reported decreased levels of most of the non-enzymatic antioxidants (i.e., vitamin C, vitamin E, reduced GSH, lycopene, and carotenoids) in asthmatic children.

Results of antioxidant intake in asthmatic subjects are conflicting. Epidemiological studies indicate that dietary intake of vitamin C may be associated with a reduced risk of asthma (Hatch, 1995; Soutar et al., 1997). On the contrary,
many studies have reported no association between asthma and vitamin C (Troisi et al., 1995; Fogarty et al., 2003). Many workers have suggested in both healthy subjects (Chatham et al., 1987; Samet et al., 2001) and asthmatic patients (Trenga et al., 2001), that antioxidant supplementation (vitamin C and vitamin E) may protect against the acute effects of ozone on lung functioning.

Dietary supplementation with vitamins E and C benefits asthmatic adults who are exposed to air pollutants (Trenga et al., 2001). Romieu et al. (2002) reported that antioxidant supplementation might modulate the impact of ozone exposure on the airways of children with moderate to severe asthma. Studies have reported decreased oxidative damage in asthmatic mice treated with antioxidants (Okamoto et al., 2006; Dittrich et al., 2009; Lee et al., 2009).

The oxidant-antioxidant imbalance in the plasma of smokers and asthmatic patients and healthy nonsmokers was assessed by comparing the Trolox equivalent antioxidant capacity (TEAC) and products of lipid peroxidation and plasma TEAC was significantly lower in patients with exacerbations of asthma compared with healthy nonsmokers, plasma TEAC was less reduced in subjects with stable asthma (Rahman et al., 1996). Katsoulis et al. (2003) also reported decreased plasma antioxidant capacity in exacerbation of asthma as compared to stable asthmatics.

Different antioxidants together by co-operation provide better defense against attack by oxidants, in comparison to an individual compound. Thus, studying overall antioxidant capacity may yield more information than measuring individual antioxidants. Also, total antioxidant power is the effect of strongly regulated antioxidant network among different antioxidants and therefore is regarded as more representative of oxidant-antioxidant balance than the concentration of single antioxidants (Chaudiere and Ferrari-Illiou, 1999; Ghiselli et al., 2000).

Ferric reducing ability of plasma (FRAP) assay, is a rapid and novel method to measure the total antioxidant power of plasma. The basic principle of this assay is the conversion of Ferric to ferrous ion at low pH and formation of a ferrous-tripryridyltriazine (TPTZ) complex which gives blue color (Benzie and Strain, 1999). FRAP analyze the ability to reduce the ferric ion. The FRAP assay measures the formed ferrous ions by increased absorbance. The assay determines the antioxidant activity in the micromolar range needing minutes to hours (Schlesier et al., 2002). The value of FRAP has been reported to be lower in asthmatic subjects as compared to
healthy individuals. Also, it has been observed to be decreasing with increases in exacerbations of asthma (Ahmad et al., 2012).

2.2.2 Catalase activity and asthma

The enzymatic antioxidants such as catalase present in the pulmonary fluid and interstitial spaces of lungs and also in the blood vessels and airways help convert the potent oxidant hydrogen peroxide (H$_2$O$_2$) to H$_2$O thus help in reducing systemic oxidant level. Catalase is a tetrameric, ubiquitous hemoprotein with a molecular weight of 240kDa that undergoes alternate divalent oxidation and reduction at its active site in the presence of its substrate, H$_2$O$_2$ and catalyzes the dismutation reaction (Deisseroth and Dounce, 1970; Chance, 1979; Reid et al., 1981).

The active site of enzyme contains a porphyrin ring in which iron is bound to four nitrogen atoms of the ring. The fifth valence position is coordinated to tyrosine 358 of catalase, while the sixth valence position is left free for interaction with substrate. The whole reaction proceeds in two steps. First, Fe$^{3+}$ reacts with hydrogen peroxide that results in cleavage of the O–O bond in H$_2$O$_2$, and the oxygen remains bound to the sixth valence position of Fe$^{5+}$, leading to formation of compound I. Compound I may oxidize a second peroxide molecule to oxygen, while the oxygen bound to the iron is released as water (Bartosz, 2005). Alternatively, Compound I may undergo inactivation by reduction to Compound II [Fe$^{4+}$] by oxidants, or from itself by formation of a tyrosyl radical (tyrosine 370) under prolonged oxidative stress.

The colorimetric method for measuring catalase (CAT) activity using K$_2$Cr$_2$O$_7$/acetic acid was described by Sinha (1972). Since then, several modifications have been proposed by various researchers worldwide. The sensitivity of catalase determination was increased at least 20 times than previous methods by means of the Clark oxygen electrode by Del Río et al. (1977). The assay was based on measurement of the initial rate at which oxygen was released by catalase in an oxygen-free buffer. Displacement of oxygen was brought about by flushing with nitrogen, and the substrate used was hydrogen peroxide at a 33.5 mm final concentration. The method is rapid and can be used with crude catalase preparations. A rapid, cost-efficient, spectro-photometric assay for serum catalase activity which was a combination of optimized enzymatic conditions and the spectrophotometric assay of hydrogen peroxide based on formation of its stable complex with ammonium molybdate was developed by Göth (1991).
Masuoka et al. (1996) developed a new method for the measurement of catalase activity by hydrogen peroxide determination and rates of hydrogen peroxide removal by erythrocytes from human subjects, rats and mice. Hydrogen peroxide was determined by converting it to the indamine dye with a water-soluble iron porphyrin and measuring the absorbance at 590 nm. A novel method for the fluorometric determination of the activity of the enzyme catalase, based on the finding that H$_2$O$_2$ in the europium (III)–tetracycline–hydrogen peroxide system is consumed by catalase was described by Wu et al. (2003).

Catalase is effective in the presence of high H$_2$O$_2$ concentrations (Cantin and Crystal, 1985), but under prolonged oxidative stress with oxidation of NADPH, catalase activity drops (Kirkman et al., 1999). However, in the presence of low concentrations of either H$_2$O$_2$ or other peroxides, the glutathione system plays a critical role (Cantin 1999). Though CAT is not essential for some cells under normal conditions, but it plays a crucial role in the acquisition of tolerance to oxidative stress. These enzymatic antioxidants have been reported to be decreased in asthmatic patients that further aid in systemic oxidative stress (Bowler, 2004).

Enzyme activity can be regulated by post-translational processes. Under oxidative stress, the Abl family of receptor tyrosine kinases causes phosphorylation of catalase at tyrosine 231 and tyrosine 386, which results in increased activity and lower cellular H$_2$O$_2$ levels (Cao et al., 2003). On the other hand, oxidation of tyrosine residues, in particular tyrosine 358, has been linked to loss of catalase activity under oxidative stress, for example, in asthma (Ghosh et al., 2006).

There are several evidences suggesting low activity of catalase in asthma subjects (Novak et al., 1991; Shanmugasundaram et al., 2001) but, some have also reported that catalase activity remains unchanged (Smith et al., 1993; Mohan and Das, 1997; DeRaeve et al., 1997; Tekin et al., 2000). Mak et al. (2004) have reported higher level of erythrocyte catalase activity in Chinese asthmatic patients. The lower activity of catalase in the red blood cells of asthmatic patients has also been reported by Rai and Phadke (2006). Ahmad et al. (2012) reported decreased catalase activity in asthmatic subjects as compared to healthy subjects and also among the asthmatic patients with increased severity of disease. They also found a negative correlation of the catalase activity with duration of the disease while a positive correlation with measured FEV1 of the subjects.
2.2.3 Lipid peroxidation and asthma

Lipid peroxides and oxygen radicals are associated with many cytotoxic reactions (Walsh, 1994; Halliwell, 1994). They cause severe damage to the biological membranes, making them leaky and ultimately causing complete breakdown (Halliwell, 1994). Malondialdehyde, a breakdown product of lipid peroxidation, affects the membrane proteins by cross-linkage, making receptors and enzymes inactive (Walsh, 1994; Halliwell, 1994).

Lipid peroxidation occurs in three steps. When a free radical attacks a lipid molecule, a carbon radical is produced that reacts rapidly with oxygen molecule to produce a peroxyl radical. This is called as chain initiation. The peroxyl radical reacts with another lipid molecule and extracts a hydrogen atom to produce a lipid hydroperoxide and a new carbon-centered radical which further reacts with another oxygen molecule to form new peroxyl radicals (chain propagation). These radicals are scavenged by antioxidants or the peroxyl radicals react to form ketones or alcohols, process called as chain termination (Halliwell and Gutteridge, 2007). Peroxidation of polyunsaturated fatty acids with two or more double bonds forms malondialdehyde (MDA) (Halliwell and Gutteridge, 2007). The concentration of the products of lipid peroxidation like malondialdehyde increases during diseased state and a great biomarker to assess increased oxidative stress (Valko et al., 2007). MDA concentrations are determined using TBARS (Thio-barbituric acid reactive substances) assay. MDA is produced as an end product of oxidation and decomposition polyunsaturated fatty acids containing three or more double bonds. MDA forms adduct with TBA which can be quantified using spectrophotometric assay. MDA is regarded as one of the most reliable biomarker for the evaluation of oxidative stress or free-radical mediated lipid peroxidation in vivo (Dalle-Donne et al., 2006).

The oxidative stress is increased in asthma which is assessed by an increased ROS production by blood monocytes, neutrophils, and eosinophils (Chanez et al., 1990; Vachier et al., 1992 and Rahman et al., 1996), increased production of lipid peroxidation products (Rahman et al., 1996) and increased plasma isoprostanes (Wood et al., 2000). The increased oxidative stress during asthma can be attributed to increased lipid peroxidation which is detected by plasma isoprostanes (Montuschi et al., 1999; Wood et al., 2000) and also by systemic level of malondialdehyde in
asthmatic subjects (Ozaras et al., 2001; Nadeem et al., 2003; Ahmad et al., 2012). Inhalation of steroids for treatment also affects the lipid peroxide levels in asthma patients. Level of lipid peroxidation reduces after treatment in asthma subjects but still remains higher than healthy subjects (Ozaras et al., 2001; Baraldi et al., 2003; Alzoghaibi and Bahammam, 2007).

Oxidative DNA damage and its relation to the oxidative stress in Saudi asthmatic patients was studied by Al-Afaleg et al. (2011). A total of 50 Saudi asthmatics and 50 healthy control subjects were enrolled in the study. All were nonsmokers and were not on vitamin supplementation. Serum levels of 8-oxo-deoxyGuanosine (8-oxo-dG), malondialdehyde (MDA) and nitrite were measured. Oxidative DNA damage marker measured by serum level of 8-oxo-dG was higher in asthmatics compared to control. Similarly, the MDA levels and nitrite were higher in asthmatics compared to control subjects. The concentration of MDA in plasma was found to be lower in asthmatics with increased severity as compared to less severe or controlled asthmatics and healthy subjects and it was positively correlated with measured FEV1 of the patients as well controls (Ahmad et al., 2012).

Though the data obtained from various reports are difficult to compare and often conflicting due to differences in disease severity, blood components and body fluids being measured, but, it is clear that overall status of antioxidants is altered in asthma implying a disrupted oxidant-antioxidant balance leading to oxidative stress. Thus, it is required to assess the relationship between oxidative stress and disease progression in asthma in order to understand the complete pathophysiology of disease and how to overcome the damage caused due to the inflammation.

2.3 Asthma and genetic damage

The ROS alter gene expression and disrupt normal repair mechanism (Poulson et al., 2000). They induce DNA damage by base modifications (purine, pyrimidine, or deoxyribose modifications), base oxidations, single or double strand breaks and formation of DNA adducts (Dizdaroglu et al., 2002; Cadet et al., 2003; Bjelland and Seeberg, 2003). Further, DNA damage can arrest or induce transcription, resulting in induction of signal transduction pathways, replication errors and genomic instability, associated with carcinogenesis (Marnett, 2000; Valko et al., 2006). Oxidative damage to DNA in buccal mucosa and lymphocytes can be measured using MN assay and comet assay as biomarkers.
2.3.1 Micronucleus (MN) assay

The micronucleus assay in exfoliated buccal epithelial cells is a less invasive and cost-effective technique to assess DNA damage in large populations in which formation of anomalous nuclei or cell is the end point to spot damage (Stich and Rosin, 1983; Holland et al., 2008). The formation of micronuclei and other cellular anomalies from either acentric chromosome fragment or a whole lagging chromosome occurs as a result of chromosome breakage due to unrepaired or misrepaired DNA strand breaks or malsegregation of the chromosomes due to mitotic malfunction. The reason behind these might be the defects in DNA repair genes, deficiency of the cofactors required for DNA metabolism, defects in the cell cycle check point or exposure to some clastogens or aneugens (Stich and Rosin, 1983; Fenech et al., 1999; Fenech, 2000; Fenech, 2005; Holland et al., 2008; Fenech et al., 2011).

Thus, MN gives a measure of both chromosomal anomaly and chromosome loss similar to classic chromosome analysis (Fenech and Morley, 1986; Muller and Streffer, 1994; Tucker and Preston, 1996; Evans, 1997; Fenech, 1997; Kirsch-Volders, 1997; Miller et al., 1997) hence; it is one of the preferred methods for detecting DNA damage at chromosome level (Fenech et al., 1999; Fenech, 2000).

MN assay was first time used in vitro by Evans et al. (1959) in radiation experiment with *Vicia faba* and in vivo by Matter and Schmid (1971) in bone marrow cell studies. Stich et al. (1982) firstly proposed the MN assay in exfoliated buccal epithelial cells and suggested that presence of MN in a cell represents an “internal dosimeter” to assess exposure to genotoxic or carcinogenic agents. Protocol for the MN assay with exfoliated buccal mucosa was developed by Stich et al. (1984). Rosin (1987) proposed protocols for Fuelgen staining techniques.

Formation of micronucleus occurs either due to (1) chromosomal breakage or (2) dysfunction of mitotic apparatus. Clastogens induce chromosome breaks and produce acentric fragments (Ford et al., 1998; Falck et al., 2002), while aneugenic agents interfere with spindle formation resulting in lagging whole chromosome behind at anaphase which get surrounded by nuclear envelope and form micronucleus (Cimini et al., 2002).

Besides MN, other nuclear anomalies are also observed and hence Tolbert et al. (1992) proposed a modified criterion for scoring MN and other nuclear anomalies.
Fenech (2000) proposed the detailed guidelines for scoring MN and other nuclear anomalies like nucleoplasmic bridges, necrotic and apoptotic cells in PBLs.

There are various modifications of MN assay which include (1) Cytokinesis blocked micronucleus assay with peripheral blood lymphocytes (PBLs), first described by Fenach and Morley (1985a and 1986) also the most common method used for genotoxicity testing worldwide, (2) MN assay with exfoliated buccal mucosa; a suitable, cost effective, easy and non-invasive approach for detecting damage (Fenech et al., 2011), (3) MN-FISH; to localize specific DNA sequences within interphase chromatin and metaphase chromosome and to identify both structural and numerical chromosome changes (Hovannisyan, 2010), (4) Live MN assay; first reported by Huang et al. (2011) using DNA binding fluorescent dyes to visualize MN in live cells.

The HUMN (HUman MN) project launched at the Seventh International Conference of Environmental Mutagenesis in Toulouse, France, September 7-12, 1997 was an attempt of obtaining the baseline data of MN frequencies from different populations worldwide, and the influence of various confounding factors on the MN frequency. It was also aimed to compare the different techniques in view of defining a standard protocol, for population based case-control studies and to determine the reliability of MN assay as a biomarker for ageing, cancer and other diseases (Fenech et al., 1999, 2011).

Because of high reliability and low cost, this technique has been successfully adopted worldwide for both in vitro and in vivo studies of genetic damage. It is also a great biomarker for early detection of cancer and carcinogenic effects of various agents (Bonassi et al., 2005). Micronuclei (MNi) and other nuclear anomalies are associated with genetic defects, ageing, genotoxicity, cardiovascular disease, neurodegenerative disorders and development of oral and other malignancies (Holland et al., 2008).

In 2007, The HUMNXL (Human MN in eXfoLiated cells) project, which is an international collaborative study on MN and other nuclear anomalies in buccal exfoliated cells was launched at the Conference on Environmental Mutagens in Human Populations, held in Antalya, Turkey with the following objectives; (1) to identify technical variables that affect the measurement of buccal MN frequency in human populations, (2) to identify lifestyle variables affecting this frequency, (3) to identify protocol variables that affect the recovery and scoring of MN, (4) to use this
information to design intra- and inter-laboratory validation studies of the method and scoring criteria, and (5) determine the role, if any, of buccal MN and other nuclear anomalies in monitoring genetic damage and prediction of cancer and other degenerative disease (Fenech et al., 2007, 2009).

A statistically strong correlation was observed between the increased MN frequency and PBLs and buccal epithelial cells in the group exposed to genotoxic compounds in a meta-analysis of 63 human populations, supporting the Buccal MN assay as a biomarker of DNA damage (Ceppi et al., 2010).

Applications

MN assay has been widely used to study the effect of lifestyle and other related factors. Various studies have demonstrated age related increase in MN frequency (Fenech and Morley, 1986; Ganguly, 1993; Bolognesi et al., 1997; Bolognesi et al., 1999). Increase in MN frequency with age has been reported in both males and females (Ganguly, 1993) but a higher frequency of micronuclei has been observed in females. This is probably due to the tendency of the inactive X-chromosome to be included in the micronuclei formation in women (Catalan et al., 1995, 1998). Hando et al. (1994) reported a ten-fold difference in the MN frequency in women as compared to men. Studies have explained that the increase in chromosomal aberrations and MN frequency with age is presumably associated with genetic damage due to accumulation of mutations and progressive impairment of DNA repair capacity (Barnett and King, 1995). Variations in MN frequency with gender and age have also been reported by Wojda and coworkers (2003, 2006, 2007).

Micronucleus assay in the exfoliated buccal epithelia can be used to demonstrate the effect of smoking in human population (Stich and Rosin, 1983; Fenech et al., 1999; Nersesyan et al., 2006; Joshi et al., 2011). Many studies have reported that various forms of tobacco and related agents such as cigarette, Betel nut and Quid and reverse smoking are associated with increase in the number of micronuclei in buccal epithelial cells (Stich et al., 1982, 1984; Majer et al., 2001; Nersesyan et al., 2006; Kamboj and Mahajan, 2007; Joshi et al., 2011). While a slightly decreased MN frequency was observed in current and former smokers as compared to non-smokers (Bonassi et al., 2003).

Bonassi et al. (2011) in a collaborative study under HUMNXL compiled a database of buccal epithelial cells of 5424 subjects from 30 various laboratories worldwide and reported that MN frequency was increased in heavy smokers
(consuming 40 cigarettes/day) and decreased with daily consumption of fruits. Data from the Human Genetics Laboratory, Department of Zoology, Kurukshetra University, Kurukshetra was also invited and included in this study.

MN in exfoliated buccal cells is also an important biomarker to study the genomic damage caused by various genotoxic compounds (Stich et al., 1983). MNi expressed in immature mice erythrocytes, which retain the nucleus until 10th hour after the final division of haemopoietic development, can be used for genotoxicity testing in pharmaceutical and chemical industry (Adler, 1984) and has been the most widely used in vivo assay for screening of chemicals (OECD, 1983; Madle and Müller, 1993). Increased MNi frequency due to exposure to mobile radiations has been reported by Yadav and Sharma (2008a) and they also reported increase in the damage with increased duration of exposure.

MN assay is an effective biomarker tool to study the genomic damage associated with diseases (Fenech et al., 1999). The assay has been widely used in prediction of cancer development (Bonassi et al., 2000). A significantly higher MN frequency has been observed in both PBLs and buccal cells of the patients with Alzheimer’s disease as compared to healthy individuals (Trippi et al., 2001; Migliore et al., 2011).

Increased MN frequency and chromosomal aberrations have been reported to be linked to the acute and chronic bacterial infections (Ortiz et al., 1997; Masjedi et al., 2000; Suarez et al., 2007) and with increased cardiovascular disease mortality (Murgia et al., 2007) and pregnancy complications (Furness et al., 2010). Significant increase in the number of binucleated cells (BNCs) was reported in patients with Down’s syndrome by Thomas et al. (2008). Nersesyan and Chobanyan (2010) reported increased MNi count in the buccal cells of polycystic ovarian syndrome patients suggesting the genomic damage associated with the syndrome.

Studies have reported the elevated frequency of MN and BN in COPD (Maluf et al., 2007), type-2 diabetes (Martinez-Perez et al., 2007), head and neck cancer (Khelifi et al., 2013) and its correlation with cancer development (Cao et al., 2011) and many other diseases. Rossnerova et al. (2011) reported no significant difference in the frequency of MN between asthmatic and healthy children but a slightly higher frequency of MN in asthmatic children in comparison with controls, and a slightly greater genetic damage in girls in comparison with boys and a similar frequency of MN in young children and older children was observed. No significant DNA damage
in asthmatic children from smoking families was observed as compared to control children from nonsmoking families. The frequencies of MN in asthmatic children classified as active smokers were significantly higher in comparison with control smoking children.

The assay with buccal mucosa cells is advantageous over other variation of the technique as epithelial cells need no stimulation to undergo mitosis, whereas lymphocytes must be stimulated (Rosin, 1992; Nersesyan, 1996; Majer et al., 2001). Further, the epithelial cells take 7-21 days to appear on the surface and exfoliate and hence the effect of recent exposure can be studied (Stich and Rosin, 1984; Sarto et al., 1990; Paetau et al., 1999). Also, these can be collected easily and is less invasive; thus it is more feasible than blood collection for the study of widely dispersed population (Holland et al., 2003); in large biomonitoring studies, especially in pediatric studies and where repeated sampling is required (Bonassi et al., 2011). Epithelial cells are the first to express the genotoxic effect of inhaled and ingested genotoxic agents and their metabolites, as they are in immediate contact with these (Fenech et al., 1999).

All the above mentioned facts make this assay the biomarker of choice for both in vitro and in vivo monitoring of genomic damage (Bonassi et al., 2005) but, there are some limitations as well, one of which is that the epithelial cells undergo degenerative process that can produce anomalies that are difficult to be distinguished from MNi (Stich et al., 1983; Sarto et al., 1987).

### 2.3.2 Single cell gel electrophoresis (SCGE)/Comet assay

The SCGE assay also known as comet assay is versatile, sensitive and one of the most widely used methods to detect DNA damage (single and double strand breaks), protection and repair at the level of individual cells (Collins et al., 1997a, 2008; Tice et al., 2000; Collins, 2004; Møller, 2006).

The comet assay is a combination of methods such as nucleoid sedimentation (Cook and Brazell, 1976) and halo assay (Roti and Wright, 1987). The first attempt to quantify DNA strand breaks directly with the cells embedded in agarose on microscopic slides was made by Rydberg and Johanson in 1978. They kept the cells under mild alkaline condition for lysis of cell membranes and organelles, and after neutralization, stained them with acridine orange. The DNA damage was assessed by measuring the ratio of green (double stranded DNA) to red (single stranded DNA)
flourescence using a photometer. The nuclei of the cells were increasingly diffused after X-irradiation, in a dose-dependent manner.

Ostling and Johanson (1984) were the first to describe a micro-electrophoretic technique to directly visualize the individual cells which were suspended in thin agarose gel and layered on microscopic slide, lysed by high salt solution, electrophoresed in neutral condition and stained with a fluorescent DNA-specific dye. During electrophoresis, the broken and relaxed fragments moved towards anode leaving the nucleus behind. Singh et al. (1988) performed electrophoresis in alkaline condition (ph>13) which allowed the DNA supercoils to relax and unwind making the detection of alkali labile sites and SSBs comparatively easier.

The cell with a damaged DNA gives the appearance of a comet, due to migration of DNA fragments towards anode, with the damaged or broken fragments of DNA in its tail and the intact nucleus in its head while, the undamaged cell can be seen as a halo. Hence, the name is given as comet assay (Olive, 1989; Collins, 1992). Electrophoresis under neutral or mild alkaline conditions to detect single strand breaks was performed by Olive et al. (1990a, b). As the continuity of long duplex molecules is not affected by SSB at neutral pH; this method is useful to detect a subpopulation of cells with varying sensitivity to drugs or radiation. They used the first image analysis programme written by Dr. Durand in 1989 for analyzing comets. They also gave the concept of tail moment as a measure of DNA migration.

Another version of comet assay, i.e. acellular assay was described by Singh et al. (1990), in which the DNA embedded in the agarose gel is treated after lysis. The slides are prepared from untreated cell and after lysis, they are rinsed and then exposed in the presence and absence of the metabolic activity, to the test compound of interest and then they are continued as per protocol. As the DNA itself, not the cell is exposed; the alteration in DNA migration corresponds to the ability of the test compound to induce damage to the DNA, independent of the cytotoxicity.

The induction of double strand breaks (DSBs) and single strand breaks (SSBs) and alkali labile lesions can be measured by variation in pH conditions. The sensitivity of the neutral assay can be increased by increasing the duration (4-5 hours) and temperature (40-50°C) of lysis. The DSBs cause the release of DNA fragments into the gel and exposure to low dose of X-rays (5Gy, equivalent to 200 DSBs per cell; Powell and McMullan, 1990) can be detected using neutral comet assay (Olive et al., 1991, 1992).
The length of time used for unwinding varies considerably across studies and among different investigators. In general, an incubation period of 20 min is used suggested by Singh et al. (1988). However, in gamma-irradiated human lymphocytes, increasing the alkali unwinding duration from 20 to 60 min greatly enhanced the extent of DNA migration (Vijayalaxmi et al., 1992).

The comet assay has been reported to be highly sensitive. 0.1 DNA breaks/10⁹ Dalton were detected (Gedik et al., 1992). The sensitivity can be further increased by treating the lysed cells with proteinase K and RNase to obtain relatively pure DNA (Singh et al., 1994). Singh et al. (1988) proposed three washes of alkali in the gel with trizma buffer for 5 min each, after electrophoresis. However, increased rinsing may be useful in situations where a high background is seen during scoring (Rojas et al., 1999).

The percentage of DNA in tail quantifies the frequency of DNA breaks which ranges from few hundred to several thousands in a cell (Ross et al., 1995). Pfuhler and Wolf (1996) detected DNA-DNA cross-links using SCGE.

Collins et al. (1997b) introduced another modification by digesting the DNA after the lysis with a lesion-specific repair endonuclease, which introduced breaks at the sites of damage and hence, any lesion for which there is an endonuclease, can be detected easily.

DNA damage in nasal respiratory epithelium from children exposed to urban pollution was studied by Calderon-Garcidueñas et al. (1997). They used the alkaline single cell gel electrophoresis assay (SCGE) for measuring DNA damage in children's nasal epithelium exposed to air pollutants. All exposed children had upper respiratory symptoms and DNA damage in their nasal cells. These findings suggest that SCGE can be used to monitor DNA damage in children's nasal epithelium.

Valverde et al. (1997) demonstrated the differences in the basal level of DNA damage between young adults from the south (exposed principally to high levels of ozone) and young adults from the north (exposed principally to hydrocarbons and particles) of Mexico City. They used the SCGE assay using three different cell types (leukocytes, nasal and buccal epithelial cells). They found an increased DNA migration in blood leukocytes and nasal cells from individuals who live in the southern part of the city compared to those living in the northern part; however, no differences were observed for buccal epithelial cells.
Another variant of comet assay known as Comet-FISH, used for the first time in human cells to localize the particular chromosome domains in interphase nuclei with their distribution in the head and the tail of the comet (Santos et al., 1997). The rate of gene-specific repair after low doses of DNA damaging agents have been measured using Comet-FISH (Horváthová et al., 2004).


After identifying the minimal standards for obtaining reproducible and reliable comet data, an expert panel at the International Workshop on Genotoxicity Test Procedures (IWGTP) held in Washington, DC, on March 25-26, 1999, proposed the guidelines for the use of comet assay in genetic toxicology (Tice et al., 2000).

Banath et al. (2001) reported that increased duration of lysis and higher pH increases the sensitivity of the comet assay for detecting radiation induced DNA damage by allowing more time for DNA unwinding and diffusion before electrophoresis.

At the 4th International Comet Assay Workshop held at Ulm, Germany, 22-25 July, 2001, the protocols for comet assay were reviewed and detailed guidelines were given for in vivo alkaline comet assay. Guidelines were given in detail, for selection of animals, conducting the experiment and the statistical analysis to achieve a consistency in carrying out the assay and reproducibility of the assay across different laboratories worldwide (Hartmann et al., 2003).

A comparison of comet assay, electron microscopy and flow cytometry to check their ability to differentiate apoptosis from necrosis was done by Yasuhara et al. (2003). They reported that in Jurkat cells, skeletal and cardiac tissue of rat, neutral comet assay is as sensitive as electron microscopy, while flow cytometry lacked sensitivity.

Morley et al. (2006) explained a new experimental approach; apoptotic cells were identified in the cells embedded in agarose before the lysis, using an Annexin-V fluorescence staining method. It was based on the ability of Annexin-V to bind to phosphatidylin serine which is translocated to the outer cell membrane at an early stage.
of apoptosis. Apoptosis and DNA damage were induced by heat treatment. After four hours of the treatment, a mixture of Annexin-V positive and negative cells was seen. The comets in Annexin-V positive cells were barely detectable, and were described as ‘ghost comets’.

The Fourth International Workshop on Genotoxicity Testing (IWGT) was held in San Francisco, California, 9-10 September, 2005. A standardized protocol for \textit{in vivo} rodent, alkaline comet assay was designed which was accepted by international regulatory agencies. Most studied parameters are the $\%$ DNA in tail, tail moment and tail length (Burlinson \textit{et al}., 2007).

Lovell and Omori (2008) said that the $\%$ tail DNA is a fitting end point and also has a defined scale from 0 to 100 $\%$ which is comparable across studies and they also recommended that 50 cells per slide should be scored to get statistically consistent results. Comet assay in rodents is a standard technique for the \textit{in vivo} assessment of DNA damage and methyl-nitroso-urea is good alternative positive control inducing DNA damage in all the tissues analyzed in various studies (Smith \textit{et al}., 2008). The human lymphocytes were exposed to different mutagens and their responses were obtained using comet assay by Weng and Knehisa (2009) and they reported differential responses by CD4+, T-cells, CD8+, B-cells and NK cells to different mutagenic agents.

The comet assay protocols and image analysis used across the laboratories were studied by Forchhammer \textit{et al}. (2010) and they found that image analysis was the main source of variation across laboratories, not the laboratory protocol.

Many studies have reported that the MN assay is a more powerful tool to detect low level of genotoxicity than comet assay (Pfau \textit{et al}., 1999; Kawaguchi \textit{et al}., 2010). But, in studied mutagens, the comet assay and MN assay were found to be identically sensitive (Kawaguchi \textit{et al}.., 2010). The protocol of SCGE was modified to study five or more samples in a single slide by Zhang \textit{et al}. (2011). Retaining the sensitivity of the assay, it improved the efficiency of the assay.

The ComNet project was launched in 2011 at the International Comet Assay Workshop in Turkey to establish the comet assay as a consistent biomarker tool for determining the relevance of DNA damage, human health and diseases (Collins \textit{et al}., 2012).
Applications

SCGE assay has been widely used as a tool to assess DNA damage and repair in different cell types in a variety of studies, viz., human biomonitoring, genotoxicology, ecological monitoring, genomic instability associated with many pathological diseases and to identify mutagens and carcinogens (Anderson et al., 1998; Garaj-Vrhovac and Kopjar, 1998, 2003; Chaubey et al., 2004; Collins, 2004; Dušinska and Collins, 2008; McKenna et al., 2008; Mohseni-Meybodi et al., 2009; Kirkali et al., 2011; Jayakumar et al., 2012).

De Méo et al. (1991) for the first time used comet assay in genotoxicity testing when the mutagenicity of the agents generated by treatment of potassium permanganate to the acidic substances were studied. Many other studies have applied comet assay to study the genotoxicity of various substances like various chemicals, airborne substances, radiations and various polycyclic aromatic hydrocarbons (Hellmann et al., 1999; Winter et al., 2004). Comet assay is a highly versatile, sensitive and reliable test and it can provide important information about the genotoxicity of the hazardous substances and risk assessment after proper standardization (Dusinska and Collins, 2008).

The assay has been used in many lifestyle and nutritional studies. The protective effect of in vivo supplements of antioxidants or antioxidant-rich foods were observed in the lymphocytes as either decreased sensitivity to H₂O₂-induced damage in vitro (Duthie et al., 1996) or a decrease in endogenous base oxidation (Pool-Zobel et al., 1997). A decrease in the H₂O₂-induced damage in the lymphocytes was observed by supplementing the cruciferous and legume sprouts to the diet of healthy people (Gill et al., 2004, 2007).

Mozaffarieh et al. (2013) suggested that the cryopreservations results in increases in the number of ssDNA breaks in leukocytes, thus affecting the results of comet assay analysis. They recommended performing the comet assay analysis on freshly prepared leukocytes for more reliable results. Collins (2014) proposed that a simple modification i.e. incorporating a digestion of DNA with a lesion-specific endonuclease, makes it possible to measure oxidised bases. The assay can be used to monitor the cellular or in vitro repair of strand breaks or oxidised bases.

Comet assay has wide applications in the studies of occupationally exposed populations as well. Many workers have used this assay to study DNA damage in a variety of occupationally exposed populations like in gas station workers (Andreoli et
al., 1997); furniture plant workers (Palus et al., 1999); cigarette factory workers (Zhu et al., 1999); rubber factory workers (Somorovska et al., 1999); battery factory workers (Groot de Restrepo et al., 2000; Fracasso et al., 2002); workers of pesticide factories (Garaj-Vrhovac and Zeljezic, 2000; Zeljezic and Garaj-Vrhovac, 2001); hospital workers exposed to X-radiations (Maluf et al., 2001) and foundry and pottery workers exposed to silica (Basaran et al., 2003). In all these studies, the exposed population showed higher genetic damage as compared to non-exposed population.

Higher genetic damage was observed in the B- and T-lymphocytes and granulocytes of the workers exposed to benzene in printing company as compared to non-exposed control individuals (Sul et al., 2002). Yadav and Sharma (2008b) studied DNA damage in the lymphocytes exposed to UV-radiations with the help of comet assay.

The comet assay in combination with some suitable organism can be used as a biosensor for monitoring the contaminations in the environment due to genotoxins e.g. echinoderm embryos were used as a model organism by Boveri (1914) and a relationship between chromosomal changes and the origin of tumours was observed. The in vitro genotoxic effect of methyl-mercury in the lymphocytes of bottlenosed dolphins (Tursiops truncates) was studied using the comet assay and a dose-response related increase in DNA breaks was observed (Betti and Nigro, 1996). The use of alkaline comet assay has revolutionized the field of genetic ecotoxicology (Jha, 2004).

The comet assay has been used worldwide in measuring DNA damage and repair. The repair of UV-induced cyclobutane pyrimidine dimers by nucleotide excision repair was studied using comet assay with endonuclease V by Collins et al. (1997b). Collins et al. (2001) studied repair of endonuclease III- or FPG-sensitive sites (oxidized purines and pyrimidines), by base excision repair using comet assay. A significantly elevated level of DNA damage was observed in the exfoliated epithelial cells of tear ducts of dwellers of high ozone areas as compared to those living in the high hydrocarbon areas using comet assay (Rojas et al., 2000).

Collins et al. (2003) observed decreased endogenous DNA oxidation, improved DNA repair activity and increased antioxidant status in the lymphocytes using the comet assay, when the diet was supplemented with kiwi fruit. The DNA in the apoptotic cells is extensively damaged and the damaged DNA shows less affinity for the DNA specific stains and hence it can be distinguished from the undamaged cells using comet assay (Ostling and Johanson, 1987; Olive et al., 1993b).
Comet assay has been used widely in various clinical studies ranging from measurement of tumour growth fraction (Olive and Banath, 1992; Olive et al., 1993a), analysis of DNA DSB rejoining capacity in vitro (Olive et al., 1993b) and measurement of tumour hypoxia (Olive et al., 1993a, 1996; McLaren et al., 1997).

The comet assay can be used to diagnose Xeroderma pigmentosum and other syndromes associated or characterized by anomalous excision repair as a deficiency in excision repair has been found in the lymphocytes of the patients with Xeroderma pigmentosum, using the comet assay (Gedik et al., 1992; Green et al., 1992).

Increased extent of DNA migration has been reported in patients with type 2 diabetes mellitus as compared with controls indicating increased oxidative damage associated with the disease (Dandona et al., 1996; Pitozzi et al., 2003; Blasiak et al., 2004; Choi et al., 2005).

Biri et al. (2002) reported increased DNA strand breakage and SCEs in women who used oral contraceptives. Kopjar et al. (2002) reported high level of DNA damage in cancer patients following administration of 5-fluorouracil, adriamycin, and cisplatin (FAP protocol), and suggested a continuous biomonitoring of cancer patients after the chemotherapy.

Increased oxidative damage has been reported in the nasal epithelium of children living in Mexico due to increased air pollution as compared to those living in less polluted towns (Calderon-Garciduenas et al., 1997).

Fortoul et al. (2003) performed Single-cell gel electrophoresis assay of nasal epithelium and leukocytes from asthmatic and non-asthmatic subjects in Mexico City. The authors estimated DNA strand breaks by use of single-cell gel electrophoresis assay on 2 different cell types (i.e., nasal epithelial cells and leukocytes) sampled from asthmatic and non-asthmatic medical students in Mexico City. The authors found that asthmatic subjects had more DNA breaks in their nasal epithelial cells than did their non-asthmatic counterparts. In contrast, asthmatic subjects had less damage in their leukocytes than did non-asthmatic individuals. These findings suggest that the hyper-reactivity of the nasal epithelium prevents systemic effects from air pollutants, as reflected by less DNA injury to leukocytes of the asthmatic group. Metaplasia was evident in asthmatics that also showed eosinophils and neutrophils as well as goblet cells and mucus at a higher frequency compared with non-asthmatics.

Genotoxic differences by sex in nasal epithelium and blood leukocytes in subjects residing in a highly polluted area were demonstrated by Fortoul et al. (2004).
Higher DNA damage in nasal cells and leukocytes was found in males as compared to females and control subjects. The percentage of squamous metaplastic changes in the nasal epithelium was also higher in males compared with females and controls. Maluf et al. (2007) reported increased DNA damage in patients with chronic obstructive pulmonary disease.

Zeyrek et al. (2009) reported that lymphocyte DNA damage level increases in children with bronchial asthma and that elevated DNA damage may be related to increased oxidative stress. However, the mechanism of this association, and whether it is direct or indirect, remains to be explored.

DNA damage in children with asthma bronchiale was studied by Hasbal et al. (2010). Before and after 8 weeks of anti-asthmatic therapy blood samples were taken, DNA strand breaks and Fpg-sensitive sites in peripheral leukocytes were determined by comet assay. DNA strand breaks and Fpg-sensitive sites in the asthma group were found to be increased as compared with control group. Levels of strand breaks, Fpg-sensitive sites and GSH were found to be decreased in the asthma group after the treatment. It implies that anti-asthmatic therapy including gluco-corticosteroids not only controls asthma but also decreases mutation risk in children with asthma bronchiale.

2.4 ABO blood group and asthma

Since the discovery of blood groups by Karl Landsteiner in 1901, there have been several studies reporting the association of various diseases with particular blood groups. The frequencies of different ABO blood groups vary across the populations, suggesting that a particular blood type confers a selection advantage (e.g. resistance against an infectious disease) (Reid and Bird, 1990) and hence, the AB antigens appear to have evolutionary significance. ABO blood group system is important in transfusion and transplantation practices and the alleles for ABO blood group are present on chromosome 9 (Mohanty, 2007).

The distribution of the ABO blood group in peptic ulcer and gastric cancer patients was studied by Airid et al. (1954), Clarke et al. (1955, 1959), Buckwalter et al. (1956), Brown et al. (1956), Doll et al. (1960), Beasley (1960), and Sharara et al. (2006). People with blood group A have been reported to be more prone to gall stones, colitis and tumors of salivary glands, pancreas as well as ovary (Jesch et al., 2007). Yadav et al. (2013) studied the ABO allele distribution in cancer patients and
healthy population of Haryana. Singh and Yadav (2015) found the association of blood group O with the chronic obstructive pulmonary disease (COPD) implying that people with blood group O are more susceptible to develop respiratory and lung disorders.

The ABO blood groups are characterized by the expression of oligosaccharides on the surface of epithelial cells built up by the glycosyltransferase enzymes (Oriol, 1995; Kauffmann et al., 1996). The glycosyltransferase coded by the ABO genes modifies the glycoconjugate expression. The terminal structure of the glycoconjugates expressed in the O blood group differs from those expressed in the other ABO phenotypes (Oriol, 1995; Schenkel-Brunner, 2000). The differences between distinct glycoconjugate profiles create different binding sites on the terminal structure of the oligosaccharide chains (Henry, 2001). Since these glycoconjugates act as potential receptors for microorganisms, those expressed in the O blood group may bind allergens and influence the immune response (Karlsson et al., 1992; Henry, 2001; Lindén et al., 2008).

There are variable findings regarding the relation of ABO blood group and asthma. Some researchers have reported non-significant correlation between ABO blood group and asthma (Denborough and Downing, 1968; Bijanzadeh et al., 2009) while, others have found a strong association of the ABO blood groups and asthma (Brachtel et al., 1979; Kauffmann et al., 1996). De la Vega et al. (1976) have reported significant difference in the distribution of blood group A and O between the asthmatics and controls. Studies have reported that people with blood group O are more susceptible to lung disorders than other blood groups. Lower lung function and higher prevalence of wheezing and asthma were observed in people with O blood group (Kauffmann et al., 1996). Various researchers have reported an association between the O blood group and asthma across the populations e.g. in Italian population (Ronchetti et al., 2001), Taiwanese children (Chen et al., 2005), European adults (Kauffmann et al., 1996) and in Najaf population (Al-Shamma et al., 2008).