Introduction
**Immune system**

It is a defensive system that has evolved in vertebrates to protect them from invading pathogenic organisms and cancer, and distinguishes between self and non-self components (Blom et al., 1998). It has two main components, the innate immune system and the adaptive immune system. Functionally responding in terms of recognition and response.

**Innate immune system**

It is also called non-specific system. It provides first line of defence against a variety of pathogens. All the pathogens have some conserved features that are recognised by the system. Its evolution dates back to more than 500 million years ago and so it is found in invertebrates, plants and vertebrates (Albert et al., 2002). The innate immune system includes four defensive barriers i.e. anatomic, physiologic, phagocytic and inflammatory.

**Adaptive immune system**

It is highly specific comprising of lymphocytes and immunoglobulins. The system has evolved much after the innate immune system and so is confined to vertebrates. It has two key features i.e. specificity and memory. It is very specific about the sequence it recognises on encountering a pathogen. A second encounter with the same pathogen produces a heightened response and is capable of recognising and selectively eliminating specific foreign organisms and molecules. It displays four characteristic attributes i.e. antigenic specificity, diversity, immunologic memory, self and non-self recognition (Albert et al., 2002).

**Immune response**

It is the body’s ability to recognise and act in response to a foreign molecule. It has two complementary systems.

**Humoral immune response**

Host defences mediated by antibodies secreted by plasma cells, lymph and tissue fluid. It protects against extracellular bacteria and foreign molecules. Encounter with a specific foreign molecule activates them and antibodies are released. It is so
called because the term humoral means extracellular fluid including plasma, lymph and tissue fluids.

**Cell mediated immune response**

It is mainly comprised of cytotoxic T-cells that play an essential role in cell mediated immune response.

**Immunoglobulins (IgGs)**

They are glycoproteins secreted by B cells on encounter with antigen. They came into light early in 1890 when Von Behring and Kitasato found an agent in the blood that can neutralise diphtheria toxin (Kantha., 1991). Later in 1939, Tiselius and Kabat were able to separate immunoglobulin from sera. They are found circulating in plasma and lymph. Lymphoid and mucosal tissue are also enriched in antibodies. They are principal component of adaptive immune response. Their evolution dates back and so they are found even in phylogenetically ancient species like shark and rays. It is a complex protein that shows high specificity to the antigen it binds. At the same time, the population of immunoglobulin is highly polymorphic and targets a wide array of antigens (Du and Flajnik., 1999; Schroeder and Cavacini., 2010).

**Antibody structure**

The credit to establish basic immunoglobulin structure goes to Gerald Edelman and Rodney Porter. A typical antibody has the following components:

**Heavy and light chain**

Each antibody has 2 heavy and 2 light chains of 50 KDa and 25 KDa respectively. Each heavy chain is linked to a light chain by inter-chain disulphide bonds and other non-covalent interactions. These interactions also link the 2 heavy chains and are important to stabilise the molecule (Edelman et al., 1969) (Fig. 1).
Disulfide bonds

There are 2 type of disulphide bonds:

1) Interchain disulphide bonds: They are found between heavy and light chain as well as between 2 heavy chains. They give structural stability to the molecule. Different immunoglobulin classes have variable number of interchain disulfide bonds.

2) Intrachain disulfide bond: Each of the polypeptide also contains intrachain disulphide bonds. There are 12 such bonds in an antibody molecule.

Variable and constant region

The heavy and light chain that form each arm of the antibody are made of variable and constant region. Variable region forms the N-terminal end whereas constant region forms the C-terminal end (Fig. 1). Variable as the name suggests is the region responsible for variations in binding specificities of antibodies. No two antibodies have similar variable region. Maximum variation is seen in a sub region within variable region called “hypervariable region” which forms the antibody binding site (Fab).

Heavy chain

It has 1 variable (V_H) and 3 or 4 constant regions (C_H1, C_H2, C_H3, C_H4) depending on the antibody class. The class with 3 constant regions has a hinge region between C_H1 and C_H2.

Light chain

It is made up of 2 regions 1 variable (V_L) and one constant (C_L). There are 110-130 amino acids in each of the variable or constant region. Antibody molecules belonging to same class have similar constant region, variable region however differs within same antibody class (Wu and Kabat., 1970; Schroeder and Cavacini., 2010).
Both light and heavy chains of an antibody molecule have distinct constant and variable regions.

**Source** Albert *et al*., 2002

### Domain

Each region within an immunoglobulin is a folded structure made of anti-parallel β sheets called domain. So a light chain has 2 domains and a heavy chain has 4 to 5 domains. Each domain is pinned by intrachain disulphide bond between conserved cysteine residues (Fig. 2 & 3). Many other proteins are found to have homologous domains and so they come under immunoglobulin superfamily.

### Classes

On the basis of differences in heavy chain constant region, there are 5 classes of immunoglobulins (Kolar and Capra., 2003) (Fig. 4). These classes show differences in their size, charge, amino acid composition and glycosylation (Davies and Metzer., 1983). Some differences exist within classes and on the basis of these differences they are further divided into subclasses. The major classes and the corresponding heavy chain type and subclasses are listed in table 1 (Janeway *et al*., 2001; Vidarsson *et al*., 2014). Different classes of immunoglobulin and their respective biological properties are listed in table 2 (Sites *et al*., 1976; Riesen., 1980; Alberts *et al*., 1983; Harlow and Lane., 1988).
Fig. 2: Immunoglobulin domains

The light and heavy chains in an antibody molecule are each folded into repeating domains that are similar to one another. The variable domains (shaded in blue) of the light and heavy chains (V_L and V_H) make up the antigen-binding sites, while the constant domains of the heavy chains (mainly C_H2 and C_H3) determine the other biological properties of the molecule. The heavy chains of IgM and IgE antibodies do not have a hinge region and have an extra constant domain (C_H4). Hydrophobic interactions between domains on adjacent chains have an important role in holding the chains together in the antibody molecule: V_L binds to V_H, C_L binds to C_H1, and so on.

Source Albert et al., 2002
Fig. 3: The structure of a typical antibody molecule

Source Janeway et al., 2001
Fig. 4: General structures of the five major classes of secreted antibody

Light chains are shown in shades of pink, disulphide bonds are indicated by thick black lines. Note that the IgG, IgA, and IgD heavy chains (blue, orange, and green, respectively) contain four domains and a hinge region, whereas the IgM and IgE heavy chains (purple and yellow, respectively) contain five domains but no hinge region. The polymeric forms of IgM and IgA contain a polypeptide, called the J chain, that is linked by two disulfide bonds to the Fc region in two different monomers. Serum IgM is always a pentamer; most serum IgA exists as a monomer, although dimers, trimers, and even tetramers are sometimes present. Not shown in these figures are intrachain disulfide bonds and disulfide bonds linking light and heavy chains.

Source Goldsby et al., 2000 (pp-91)
Table-1
List of various antibody classes, heavy chain type and subclasses

<table>
<thead>
<tr>
<th>Antibody class</th>
<th>Heavy chain type</th>
<th>Subclasses</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>γ</td>
<td>4 (IgG1, IgG2, IgG3, IgG4)</td>
</tr>
<tr>
<td>IgA</td>
<td>α</td>
<td>2 (IgA1, IgA2)</td>
</tr>
<tr>
<td>IgM</td>
<td>µ</td>
<td>-</td>
</tr>
<tr>
<td>IgD</td>
<td>δ</td>
<td>-</td>
</tr>
<tr>
<td>IgE</td>
<td>ε</td>
<td>-</td>
</tr>
</tbody>
</table>

Table-2
Immunoglobulin classes and their biological properties

<table>
<thead>
<tr>
<th>Properties of different classes of immunoglobulins</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
<th>IgD</th>
<th>IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin subunits</td>
<td>1</td>
<td>1 or 2</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Antigen binding site</td>
<td>2</td>
<td>2 or 4</td>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mol wt.</td>
<td>150 KDa</td>
<td>160 or 320 KDa</td>
<td>900 KDa</td>
<td>180 KDa</td>
<td>200 KDa</td>
</tr>
<tr>
<td>Sera conc. (mg/ml)</td>
<td>10-16</td>
<td>1 - 4</td>
<td>0.5 - 2</td>
<td>0-0.4</td>
<td>0.001-0.4</td>
</tr>
<tr>
<td>Percent of total immunoglobulins</td>
<td>75</td>
<td>15</td>
<td>10</td>
<td>0.2</td>
<td>0.002</td>
</tr>
<tr>
<td>Glycosylation (by weight)</td>
<td>3 %</td>
<td>10 %</td>
<td>12 %</td>
<td>13 %</td>
<td>12</td>
</tr>
<tr>
<td>Distribution</td>
<td>Intra and extravascular</td>
<td>Intravascular and secretions</td>
<td>Mostly intravascular</td>
<td>Lymphocyte surface</td>
<td>Basophils and mast cells in saliva and nasal septum-</td>
</tr>
<tr>
<td>Function</td>
<td>Secondary response</td>
<td>Protect mucus membranes</td>
<td>Primary response</td>
<td>Unknown</td>
<td>Protect against parasites</td>
</tr>
</tbody>
</table>

Antigenic determinants

The site on an antigen that is recognised and bound by a particular antibody, TCR-peptide, MHC complex or TCR-ligand-CDI complex, is referred to as antigenic
determinant. It is also called as an epitope. Due to structural complementarily with its respective antigen, it fits into antibody in a similar way as key fits into a lock (Fig. 5). There are 3 categories of antigenic determinants:

**Isotypic determinants**

Isotypic determinants are found in the constant region of heavy chain and are similar in a particular heavy chain type within a species. However, same antibody class in different species may have differences in isotypic determinants. It is isotypic determinants that are recognised as foreign when antibody of a species is transferred to other leading to induction of immune response.

**Allotypic determinants**

Unlike isotypic determinants which are similar in a particular immunoglobulin class of all members of a species, allotypic determinants are different in different members of same species. This is because certain isotypic genes have multiple alleles. These allelic variation within members of a species generate allotypic variations. They are found in all four IgG subclasses, one IgA subclass and κ light chain type.

**Idiotypic determinants**

The set of antigenic determinants in the variable region of antibody is called idioype. There are multiple idiotypes in the variable region of each antibody. It can be located in either hypervariable regions or framework regions (Goldsby et al., 2000).

**General Functions of Immunoglobulins**

1. **Antigen Binding**: It is the primary and most important function of antibody. Antigen binding site is formed by combination of variable heavy and light chain. The site can be either a small cleft, shallow groove or deep groove depending upon the type of antigen. An efficient binding depends upon affinity of antigen and antibody. Higher the affinity of antibody, more stable is the association. The number of antigen binding sites vary in different immunoglobulin classes. IgG, IgD and IgE have 2 such sites, IgA can have either 2 or 4 antigen binding site whereas, IgM has 10 antigen binding sites (Cisar et al., 1975; Rudikoff et al., 1982).
Fig. 5: Antigenic determinants

Because antibodies have two identical antigen-binding sites, they can cross-link antigens. The types of antibody-antigen complexes that form depend on the number of antigenic determinants on the antigen. Here a single species of antibody (a monoclonal antibody) is shown binding to antigens containing one, two, or three copies of a single type of antigenic determinant. Antigens with two antigenic determinants can form small cyclic complexes or linear chains with antibody, while antigens with three or more antigenic determinants can form large three-dimensional lattices that readily precipitate out of solution. Most antigens have many different antigenic determinants, and different antibodies that recognize different determinants can cooperate in cross-linking the antigen (not shown).

Source Albert et al., 2002
2. Neutralisation: The ability of antibody to neutralise infection of susceptible cells or the toxic effect of pathogenic organisms; ultimately leading to prevention or clearance of infection, is called neutralisation reaction. Antibody binds to surface epitopes on pathogenic organism that binds to host cell and mediate harmful reactions. The binding of antibody to these epitopes prevent organisms to target host cell, thus neutralising their effect (Forthal., 2014).

3. Agglutination reaction: In these reactions, antibody binds to surface exposed antigenic determinants on bacteria or RBC, bridge them together causing agglutination (Flegel., 2015).

4. Antibody as cell surface receptors: Cell membrane bound antibodies function as receptors. B lymphocytes have antibody receptors on them. The receptors are attached such that variable region faces outside and constant region spans the membrane. Each B-cell express around $10^5$ antibody molecules of single specificity. When an antigen binds to surface antibody, a series of reactions occur that causes cell activation and differentiation (Lanzavecchia., 1985; Albert et al., 2002).

5. Complement activation: Activation of complement is a very important function of antibody. Antigen-Antibody complex activates complement system via classical pathway. Components of complementary system binds to the Fc portion of antibody and initiates a cascade of events that results in antigen destruction (Janeway et al., 2001).

6. Fc receptor binding: Antibody also mediates protective function by binding to receptors that recognise Fc portion of immunoglobulin. For different classes and subclasses different Fc receptors are available. IgG has 3 type of Fc receptors: FcγRI, FcγRII and FcγRIII. The former binds with high affinity to monomeric IgG whereas the latter two binds to IgG forming immune complexes. These receptors are found on phagocytic cells and help antibody to perform its effector functions (Ravetch and Kinet., 1993; Baudino et al., 2008).

7. Transcytosis: The transfer of immunoglobulin across epithelial surfaces to mucosal layers of respiratory, gastrointestinal and urogenital tracts is called transcytosis. The major antibody that is found in mucosal surfaces is IgA. Thus IgA is called secretory antibody (Corthesy., 2013).
8. Passive immunisation : By passive immunisation, foetus gets immunity against infections. IgG is capable of transfer across placental surface and provide passive immunity to foetus (Niewiesk., 2014).

9. Antibody as drugs : Antibodies used in pharmacy are gaining recognition. Antibodies are very useful as drugs. It has been found that they are effective in controlling diseases like cancer, allergy, autoimmune diseases and inflammation. The reason for the entry of antibodies into pharmaceutical industry is because of its high specificity to target antigen. They are efficiently used to carry therapeutic antibody to the target site resulting in reducing side effects. OKT-3 was the first antibody drug that gained approval in 1986. Till now more than 23 monoclonal antibody drugs are in market (Roque et al., 2004; Pavlou and Belsey., 2005; Wang et al., 2007).

Immunoglobulin G (IgG)

It is most abundant of all immunoglobulin classes making 80 % of total immunoglobulin pool of the serum and 10-20 % of all plasma proteins. Like other immunoglobulins, it is made by the assembly of 4 polypeptide chains. Two identical heavy and light chains of 50 KDa and 25 KDa respectively assemble to form a Y shaped molecule. It is the disulphide bonds that impart stability to H-L dimer. Each heavy and light chain is divided into variable and constant region. It is the variable region of heavy and light chain that associate to form antigen binding site. Light chain has 2 domains, V_L and C_L whereas heavy chain is made up of 4 domains , 1 variable (V_H) and 3 constant (C_H1, C_H2 and C_H3) and a hinge region between C_H1 and C_H2. These heavy and light chains combine to form 3 fragments, also called arms. The fragment that is responsible for antigen binding is referred to as antigen binding fragment (Fab), whereas the fragment responsible for imparting effector function is called crystallisation fragment (Fc). Each IgG has 2 Fab and 1 Fc fragments (Vidarsson et al., 2014). Based on the differences in constant region IgG has 4 subclasses i.e. IgG1, IgG2, IgG3 and IgG4 (Schur., 1988). Light chains are of 2 types κ and λ, with former more abundant. These IgG subclasses can pair with either κ or λ light chain. The variable region of IgG further has 2 regions i.e. hypervariable region (region with high amino acid sequence variation) and framework region (less sequence variation). The variable part of each chain has 3 hypervariable regions and 4
framework regions. It is the hypervariable region of heavy and light chain that forms antigen binding site. Framework region forms β barrel and support the loops of hypervariable region (Kabat et al., 1978; Wade and Scanlan., 1997) (Fig. 6).

**Fig. 6:** Schematic diagram of structure of immunoglobulins derived from amino acid sequencing studies

Each heavy and light chain in an immunoglobulin molecule contains an amino-terminal variable (V) region (aqua and tan, respectively) that consists of 100–110 amino acids and differs from one antibody to the next. The remainder of each chain in the molecule—the constant (C) regions (purple and red)—exhibits limited variation that defines the two light-chain subtypes and the five heavy-chain subclasses. Some heavy chains (γ, δ and α) also contain a proline-rich hinge region (black). The amino-terminal portions, corresponding to the V region bind to antigen; effector functions are mediated by the other domains. The μ and ε heavy chains, which lack a hinge region, contain an additional domain in the middle of the molecule.

**Source** Goldsby et al., 2000
**Introduction**

**IgG subclasses**

There are 4 subclasses of IgG that came into light in 1960 after extensive studies using antisera from rabbits against myeloma protein of human IgG (Schur., 1988) (Fig. 7). These subclasses in accordance with decreasing abundance are IgG1 > IgG2 > IgG3 > IgG4. They have 90% identity at the amino acid level however, structural differences cause differences in their biological activities like complement fixation, half-life, immune complex formation and placental transfer (Vidarsson et al., 2014). IgG1 mainly targets soluble protein antigens and membrane proteins. As IgG1 is most abundant, lack of IgG1 causes hypogammaglobinemia (Ferrante et al., 1990; Agarwal and Cunningham-Rundles., 2007). IgG2 is also called anticarbohydrate antibody as it binds to bacterial capsular polysaccharide. So, deficiency of IgG2 can cause bacterial infections (Siber et al., 1980; Barrett and Ayoub., 1986; Schauer et al., 2003). IgG3 is mainly involved in the induction of effector functions (Leoh et al., 2015). IgG4 (along with IgE) is secreted in response to allergens (Hammarstrom and Smith., 1983; Van de Veen and Akdis., 2016).

![Fig. 7: The 4 subclasses of human IgG](image)

**Source** Vidarsson et al., 2014
Hinge region

It is the flexible linker arm between Fab and Fc fragment. It is the length and flexibility of the hinge region that decides the conformation of Fab arms in relation to Fc arm (Rayner et al., 2015). Different IgG classes have differences in hinge region that affect their function (Michaelsen et al., 2009). IgG1 has 15 amino acid long hinge region and is very flexible (Vidarsson et al., 2014). IgG2 has 12 amino acid long hinge region and has restricted flexibility due to rigid polyproline helix (Einarsdottir et al., 2014). IgG3 has longest hinge region of 62 amino acids with 21 proline and 11 cysteine residues, presence of proline limits the flexibility. Due to long hinge region Fab is far from Fc, length of hinge region also increases molecular weight of this subclass of IgG (Saluk and Clem., 1971; Michaelsen et al., 1993). The hinge region of IgG4 is 12 amino acid long (Roux et al., 1997). All 4 IgG classes have different number of disulphide bonds in their hinge region (Hamilton., 1987; Michaelsen et al., 1993; Roux et al., 1997; Carrasco et al., 2001; Vidarsson et al., 2014).

Glycosylation in IgG

Glycosylation is essential for proper functioning of IgG. It is estimated to have 2.8±0.4 oligosaccharide molecules/mol of IgG (Rademacher and Dwek., 1983; Wright and Morrison., 1997). Most of the glycosylation occurs at asparagine 297 of Fc portion of IgG, which is conserved for glycosylation (Krapp et al., 2003). In addition, 15-20 % of normal polyclonal IgG molecules bear N-linked oligosaccharides in the variable (V) regions of the light (L) and/or heavy (H) chains. In addition to this, Fab fragment also bear 15-20 % of N-linked glycosylation sites. The N-glycans associated with Fab influence the avidity and affinity of antibodies for antigens i.e immunomodulation (Mimura et al., 2007; Boonadt et al., 2014). The composition and position of glycans is critical for proper functioning of antibody (Maverakis et al., 2015). The main sugar residues found in IgG are N-acetyl glucoseamine and mannose. Besides, fucose, galactose and sialic acid are also reported to be present (Viddarson et al., 2014).

Free radical

A free radical may be defined as chemical species possessing one or more unpaired electron in their outer orbital (Rahman., 2007). They are highly reactive
entities present in human body in minute concentrations (Cheeseman and Slater, 1993; Lobo et al., 2010; Mostafa Abd El-Aal, 2012).

A free radical can be possibly formed by any of the following ways:

1. Normal molecule undergoing homolytic cleavage produces two free radicals with each molecule having one unpaired electron.
2. By the loss/addition of a single electron from a molecule.
3. Enzymatic and non-enzymatic reactions.

Under normal circumstances, the body keeps a check on the production of free radicals. However, under some abnormality/pathology excessive production takes place. This in turn damages the cells and tissues (Wilson, 1997). The effect of these radicals is positive if produced within limit, however when the limit exceeds beyond physiological, they start attacking self-components (Lobo et al., 2010). The damage is measured in terms of peroxidised lipid components, carbonyl content, serum malondialdehyde level, free sulphhydryl content and various other assays (Dalle-Donne et al., 2003; Halder and Bhattacharyya, 2014; Noh et al., 2014, Singh et al., 2014; Mateen et al., 2016) The most important of all the free radicals are the “Reactive oxygen species (ROS)”.

**Reactive oxygen species (ROS)**

ROS are highly reactive oxygen based entities produced as normal products of cellular metabolism. They are small molecules and their action is diffusion limited, thus making their subcellular location important (Sharma et al., 2012). Reactions of molecular oxygen produces a variety of ROS such as hydroxyl radical (OH’), superoxide (O2 ’), peroxynitrite (ONOO’), lipid peroxide (LOO) as well as non-radical species like hydrogen peroxide (H2O2), hypochlorous acid (HOCl), oxides of nitrogen (NOx), ozone (O3) and singlet oxygen (1O2) among others (Uttara et al., 2009) (Fig. 8). Various normal metabolic processes such as mitochondrial respiration as well as activity of enzymes like NADPH oxidase, myeloperoxidase, xanthine oxidase, cyclooxygenase, cytochrome P450 contributes to ROS production. They mediate release of cytochrome C and other apoptotic factors by opening permeability
transition pore of mitochondrial membrane (Elahi et al., 2009). They are secreted by neutrophils and macrophages during inflammatory processes and play a role in defense mechanism, however overproduction may lead to irreversible cellular damages (Moncada et al., 1991; Babior., 2000).

**Fig. 8: Electron structures of common reactive oxygen species**

Each structure is provided with its name and chemical formula. The red • designates an unpaired electron.

*Source* http://www.biotek.com/resources/articles/reactive-oxygen-species.html

**Types of ROS**

**Superoxide (O$_2^-$)**

Single electron reduction of O$_2$ produces superoxide

$$O_2 + e^- \rightarrow O_2^-$$

All aerobic cells produce this moderately reactive oxygen species by a number of enzymatic and non-enzymatic reactions (Gill and Tuteja., 2010). NADPH oxidase, xanthine oxidase and cytochrome P-450 are the main enzymes involved in O$_2^-$ production (O’Brien et al., 2009) whereas, non-enzymatic sources include electron leakage from the redox centres to oxygen in mitochondrial electron transport chain (Turrens., 2003), coenzymes and prosthetic groups mediated transfer of single electron to oxygen atom (Turrens., 2003) and reduced xenobiotics (Lagrange et al.,
1994). It is an important ROS produced during inflammation by phagocytic cells such as neutrophils, macrophages, eosinophils and monocytes with an established role in a variety of physiological and pathological processes (Yagisawa et al., 1996; Karbownik and Lewinski., 2003; Cross and Segal., 2004). These cells on encountering foreign particles or immune complexes undergo respiratory burst releasing $O_2^-$ where they impart microbicidal function (McPhail et al., 1981; Halliwell et al., 1992; Russell et al., 2009). It is a precursor molecule which through redox reactions forms other potent ROS like ONOO$^-$ and highly reactive OH$^-$ formed by Fenton’s reaction (Liu et al., 1994; Thomas et al., 2009). Besides, it is also involved in propagation of a variety of chain reactions. At low pH, it is present in its protonated form, perhydroxyl that diminishes to < 1 % at physiological pH (Cheeseman and Slater., 1993). Diffusion controlled reaction of $O_2^-$ with nitric oxide (NO) forms highly reactive molecule peroxynitrite (ONOO$^-$) (Fridovich., 1997; Leonard et al., 2000; Ahmad et al., 2013; Ahmad et al., 2015).

**Hydrogen peroxide (H$_2$O$_2$)**

Two superoxide radicals ($O_2^-$) combine to form this moderately reactive molecule. It is a colourless liquid with a half-life of 1 ms (Bhattacharjee., 2005). This oxidising agent is a source of highly reactive OH$^-$ via the Fenton's type reaction (Cave et al., 2006; Thomas et al., 2009). As per estimates, perfused livers from normally fed rat have an H$_2$O$_2$ production rate of 82 nmol/g of tissue/min (Chance et al., 1979). Micromolar concentrations of H$_2$O$_2$ are found in lens of human eye and expired human breath, possibly released by oral bacteria (Bhuyan and Bhuyan., 1977; Williams and Chance., 1983). Several bacterial species, spermatozoa, phagocytic cells release H$_2$O$_2$. In vitro, its production is reported from mitochondria, chloroplast and microsomes (Chance et al., 1979; Halliwell., 1981). It has a wide range of applications, its oxidising ability makes it a useful antiseptic, it inhibits microbial growth (Woo et al., 2003). It acts as a bleaching agent, and thus bleaches wood pulp to make white paper and melanin of hair (Lopez et al., 2003; Liu et al., 2013). It prevents corrosion by destroying agents such as residual chlorine, reduces sulphur compounds and sulphites that when applied to equipments form corrosive acids on exposure to air. Biological oxygen demand (BOD) and chemical oxygen demand (COD) contributing organic and inorganic pollutants can also be oxidised by H$_2$O$_2$ (Prashant and Malu., 2013). In mammalian cells, it is produced by several metabolic
Introduction

pathways like end steps of purine degradation catalysed by xanthine oxidase, NADPH oxidase, mitochondrial electron transport chain and arachidonate pathways. Endothelial cells also release ROS during infection and trauma. ROS in turn mediates the release of inflammatory mediators such as tumour necrosis factor alpha (TNF-α), interleukin 8 (IL-8) and neutrophil binding adhesion molecule which promote endothelial cell binding to neutrophils. The cascade promotes further release of ROS causing wound healing (DeForge et al., 1993; Lo et al., 1993; Marui et al., 1993).

Hydroxyl radical (OH•)

It is a highly reactive species of activated oxygen with a short life time of 10^{-9} to 10^{-11} seconds (Mostafa Abd El-Aal., 2012). It is mainly produced during monovalent reduction of oxygen molecule in plants, animals and microorganisms (Halliwell and Gutteridge., 1989; Halliwell et al., 1992; Sharma et al., 2012). It reacts at diffusion controlled rates (limited to a few nanometers from the site of generation) (Hippeli and Elstner., 1997), with most of the biological macromolecules like sugar, amino acids, DNA, organic acids, phospholipids (Nagy and Floyd., 1984; Cheeseman and Slater., 1993; Nimse and Pal., 2015). This high reactivity makes the radical responsible for oxygen toxicity in vivo (Gill and Tuteja., 2010). The release of OH• increases under stressful conditions targeting biomolecules which in turn causes variety of cellular disorders like inflammation, cell death and embryo teratogenesis (Winterbourn., 1981; Babbs et al., 1989; Desesso et al., 1994). Excessive OH• production can play a major role in pathogenesis of numerous diseases like carcinogenesis, Parkinson’s disease, hemochromatosis, diabetes and associated complications along with RA (Ohkuwa et al., 1995; Kaur et al., 1996; Giasson et al., 2000; Dawson and Dawson., 2003; Anderson et al., 2008). In biological systems it is produced mainly during Fenton’s reaction involving H_2O_2 and Fe^{2+}.

\[
Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^- \tag{1}
\]

\[
Fe^{3+} \text{ reacts with superoxide ion (O}_2^- \text{) regenerating Fe}^{2+}. \nonumber
\]

\[
Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \tag{2}
\]

Equation 1 and 2 together are called Haber Weiss reaction

\[
H_2O_2 + O_2^- + Fe^{2+}/Fe^{3+} \rightarrow OH^- + OH^- + O_2 \tag{3}
\]
Introduction

Mitochondria are the main site for Fenton’s reaction as it possess all the precursor components involved in reaction \( \text{O}_2^-, \text{H}_2\text{O}_2, \text{Fe}^{2+} \). Complex I and III within the mitochondrial matrix generate \( \text{O}_2^- \) that reacts with manganese (Mn) containing superoxide dismutase forming \( \text{H}_2\text{O}_2 \). \( \text{O}_2^- \) can also induce iron release from Fe-S centers in complex I and II during stressful conditions. Since, the two prerequisites for the generation of \( \text{OH}^- \) are reducing condition and labile iron pool; iron overload is directly related to disease pathogenesis. Iron chelators such as desferrioxamine reduce \( \text{OH}^- \) mediated damage by preventing Fenton’s reaction (Chen and Schopfer, 1999; Thomas et al., 2009).

Singlet oxygen (\( ^1\text{O}_2 \))

Since this ROS does not have any unpaired electron, its reactivity is associated with elevation of electron to higher energy level. Like other ROS, it is also capable of causing tissue damage (Devasagayam and Kumat, 2002). Its cytotoxicity is seen in eukaryotic cells, bacteria and viruses (Dahl et al., 1987; Piette, 1991; Devasagayam and Kumat, 2002). Genotoxic effects on eukaryotic cells are associated with extracellularly generated \( ^1\text{O}_2 \) (Epe., 1991). This ROS of short lifetime (3 \( \mu s \)) has a prudent role in tumour induction, skin photosensitivity, erythropoietic porphyria and lung oxidant injury (Cadenas and Sies, 1984; Eisenberg et al., 1984; Sies, 1986; Vallyathan and Shi, 1997; Skovsen et al., 2005). Photosynthetic cells have high concentration of \( ^1\text{O}_2 \) where it is formed on reaction of triplet state of chlorophyll (resulting from insufficient dissipation of energy during photosynthesis) with oxygen (Hatz et al., 2007).

Oxidative stress

An intact pro-oxidant/antioxidant system within the cells to detoxify oxidants is found in human body. Oxidative stress is defined as a condition in which a shift occurs in the level of pro-oxidant/ anti-oxidant in favour of the former. The balance is disturbed either due to excessive ROS formation or a decline of antioxidants in the body. It is not only the endogenous synthesis but also a number of exogenous sources that contributes to elevation in the level of ROS. All biological macromolecules like protein, carbohydrate, nucleic acids are potentially damaged due to free radical attack. ROS has an established role in numerous diseases like diabetes, RA, skin diseases,
neurodegenerative diseases, cardiovascular diseases (Halliwell et al., 1992; Poljsak et al., 2011; Poljsak and Milisav., 2012; Kruk and Duchnik., 2014).

**Formation of free radical**

**Endogenous formation**

Endogenous free radical sources include production and action within the cell or outside the cell. Free radical production can either be accidental or deliberate. Its production is beneficial if constrained and targeted. Accidental production is kept limited by the high efficiency of electron transport chain and tight sequestration of metal ions (Palmieri and Sblendorio., 2006). Amongst a variety of free radicals, O$_2^•$- is the primary ROS produced by a single electron reduction of O$_2$. It is a by-product of various metabolic pathways operative in chloroplast, mitochondria, endoplasmic reticulum and peroxisomes. Maximum production occurs from the mitochondrial electron transport chain (Turrens., 2003; Boguszewska and Zagdanska., 2012; Kalogeris et al., 2014). Besides, auto-oxidation of semiquinones on the internal mitochondrial membrane also generate O$_2^•$- (Valdez et al., 2000). It is also released during respiratory burst from macrophages and neutrophils as part of their oxidative killing mechanism role by NADPH oxidase system (Robinson., 2008). Auto-oxidation of certain compounds like ascorbic acid, adrenaline, flavin coenzymes and thiols also generate O$_2^•$- (Palmieri and Sblendorio., 2006). Enzymes such as xanthine oxidase, tryptophan dioxygenase, indole aminedioxygenase, flavin oxidases, cyclooxygenases, peroxidases produce O$_2^•$- directly. O$_2^•$- can be spontaneously converted to H$_2$O$_2$ by the action of superoxide dismutase (SOD). Enzymes like monoamine oxidase, L-amino acid oxidase is involved in direct formation of H$_2$O$_2$ (Cohen and Kesler., 1999; Minibayeva et al., 2001; Armstead., 2003; Lukasheva et al., 2012 ). O$_2^•$- can also transform into more reactive free radical species like peroxyl radical (O$_2^•^2$), alkoxyl radical (RO), hydroxyl radical (OH\textsuperscript{+}) (Sharma et al., 2012). OH\textsuperscript{+} can also be formed by the reaction of O$_2^•$- with H$_2$O$_2$ in presence of Fe$^{2+}$. The reaction of H$_2$O$_2$ with chloride forms hypochlorous acid (HOCl) which then react with amino acids forming chloramines. Similar reaction take place with other halides (Chen and Schopfer., 1999; Pullar et al., 2000). Another highly reactive free radical peroxynitrite (ONOO\textsuperscript{-}) is formed when O$_2^•$- reacts with NO. NO is a signalling molecule produced from various sources one being nitric oxide synthase (NOS) catalysed deamination of
L-arginine to L-citrulline in presence of NADPH (Tuteja et al., 2004; Szabo et al., 2007; Habib and Ali, 2011). It reacts with various biomolecules in human body fluids and tissues forming nitro-tyrosine whose accumulation in human brain causes neurodegenerative diseases (Dawson and Dawson, 1998).

**Exogenous formation**

The main exogenous source of free radicals is environment. Environmental sources include ionising radiation from industry, cosmic rays, medical X rays, solar energy. Gases mainly ozone (O$_3$) and nitric oxide (NO) from automobile exhausts, cigarette smoke, transition metals (cadmium, mercury, lead, asbestos, iron), certain drugs (cyclosporine, bleomycin, gentamycin, tacrolimus, halothane, paracetamol), unsaturated fat, cooked food and a variety of other chemical compounds from food, water and air. Through these environmental sources free radicals can easily penetrate into the body (Schuchmann and Laidler, 1972; Pham-Huy et al., 2008; Phaniendra et al., 2015).

**Free radical mediated damage to biological macromolecules**

ROS are highly toxic and its excessive production is very harmful for biological macromolecules (Fig. 9). ROS both from exogenous and endogenous sources target biomolecules comprising of lipids, proteins, nucleic acids as well as extracellular matrix components like collagen and proteoglycans (Uttara et al., 2009; Gill and Tuteja, 2010; Hardin et al., 2015). Free radicals react with these biological components and in turn generate secondary radicals to initiate a vicious chain reaction. This leads to impairment of cellular function, disease development, disease progression and ultimately cell death (Dalle-Donne et al., 2006). These ROS cause undesirable oxidation of macromolecules (fragmentation, crosslinking, aggregation) forming lipid peroxidation products (Ayala et al., 2014), protein and DNA oxidation products (Shulaev and Oliver, 2006; Cadet and Wagner, 2013). Free radical mediated damage to enzyme cause inhibition and denaturation of enzyme function (Stadtman et al., 2003). A variety of diseases such as atherosclerosis, neurodegenerative diseases, diabetes and associated complications, rheumatoid arthritis has free radical chemistry behind them (Uttara et al., 2009). Thus to avoid excessive production and its associated damage, it is crucial to keep a check on the
level of free radicals in tissue and biological fluids. A variety of biomarkers of oxidative and nitrosative stress gives a clear indication of location and amount of these species in the body. But hand in hand these assays have sensitivity and specificity limitations also (Ogino and Wang., 2007).

Fig. 9: Reactive oxygen metabolism

Under normal conditions ~5% of respired oxygen is metabolised to water via this path. Superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) are produced normally and may have protective actions. However with ischemia and reperfusion the normal balance is lost and hydroxyl radical (OH$^-$) can be produced via the Fentons reaction.

Source Baeker., 2004

Damage to DNA

DNA is an important and vulnerable target of ROS formed in its vicinity as part of cellular metabolism. Free radicals target both bases and sugar phosphate backbone and till now more than 20 base lesions are known, the most important being 8-oxo-2’deoxyguanosine (Halliwell and Gutteridge., 1999; Cooke et al., 2003). The reaction results in extensive strand breakage, deletion and modification of bases as
well as formation of dimers (more likely when the adjacent pyrimidine bases are exposed to UV-B radiation) (Tuteja and Tuteja., 2001). Amongst all reactive oxygen species, OH’ is highly damaging that mainly targets the double bond of DNA bases, the most susceptible being C-4 and C-5 of pyrimidine and C-4, C-5 and C-8 of purine (Cadet and Wagner., 2013). It also reacts by abstraction of hydrogen atom from methyl group of thymine (forming allylic radical) or any carbon of sugar phosphate backbone (O’Neill and Chapman., 1985; Sonntag., 1987; Steenken., 1987; Vieira and Steenken., 1990). Similar to OH’, O₂⁻ also has high reactivity and mainly targets guanine residue, NO causes deamination of nucleotides. H₂O₂ and O₂⁻ are least reactive of all (Wiseman and Halliwell., 1996; Hitchon and El-Gabalawy., 2004). The physiological effect of DNA damage is reduced synthesis of protein, cell membrane destruction, cell death, DNA-protein cross links (Britt., 1999; Borges et al., 2008).

Oxidative modification of DNA has cytotoxic, mutagenic and cytostatic effects, if unrepaired. A variety of repair mechanisms operate to rectify the effect of oxidative damage. Still, many base remain unrepaired and so, the oxidative base level of DNA is actually the unrepaired bases. It has been reported that H₂O₂ mediated damage to DNA supress repair mechanism. (Cheeseman and Slater., 1993; Cooke et al., 2003).

**Damage to protein**

ROS mediated structural damage to protein has detrimental effects inducing functional compromise, cell toxicity and disease pathogenesis (Kurien and Scofield., 2008). Protein modification may occur either by direct amino acid oxidation or secondary attack via lipid peroxidation products like 4-hydroxynonenal (HNE) and malondialdehyde (MDA) (Uchida and Stadtman., 1994; Requena et al., 1997b). The oxidised proteins are distributed randomly both inside and outside the cell (Pajares et al., 2015). Most likely targeted amino acids include cysteine, methionine, arginine, lysine, histidine, threonine, valine, tyrosine and tryptophan (Grimsrud et al., 2008; Phaniendra et al., 2015). Cysteine and methionine (sulphur containing amino acids) upon oxidation form disulphide bonds and derivatives of sulfenic acid (-SOH), sulfinic acid (SO₂H) and sulfonic acid (-SO₃H). Disulfide bond formation occurs through an intermediate thiyl radical (-S˙), formed by abstraction of H₂ atom from a cysteine residue (Mozziconacci et al., 2008; Kettenhofen and Wood., 2010). ROS mediated disulphide bond formation is explained for actin and Yap 1 transcription factor causing reduced dynamics of actin which further depolarises mitochondrial
membrane (Costa et al., 2007). Protein carbonylation, a widely used marker for protein oxidation has undesirable effects. A number of amino acids undergoing oxidative modifications give carbonyl groups. Carbonylation may cause protein unfolding, increased hydrophobicity, functional impairment, altered activity and aggregation of protein; thus increasing their susceptibility towards proteolytic attack (Dalle–Donne et al., 2006). The damaged proteins are hard to repair and are destined for degradation by cellular proteolytic system. More severe oxidation, crosslinking and complications result, if early removal is failed. These unremoved proteins form aggregates which open up way for diseases and aging (Costa et al., 2007). A large number of enzymes such as serum acetylcholine esterase, alkaline phosphatase, carbamoyl phosphatase synthetase, glucose-6-phosphate dehydrogenase and muscle phosphoglucomutase are prone to ROS mediated inactivation (Shinar et al., 1983; Deshpande and Joshi., 1985; Alonso et al., 1992; Mordente et al., 1987; Szweda and Stadtman., 1992). The chances of inactivation increase manifold if the target amino acid is in close proximity of active site/metal binding site of enzyme; for instance, modification of single arginyl and histidyl residue in glutamine synthetase and histidyl residue in Cu²⁺/Zn²⁺ SOD located adjacent to metal binding site results in inactivation of enzyme (Levine., 1983; Climent et al., 1989; Yim et al., 1990; Liaw et al., 1993). ROS can also target enzyme cofactors as in mitochondrial aconitase, one of the free radical $\cdot O_2$ results in release of Fe from 4Fe-4S cluster causing detrimental effects in the respiratory function due to loss in enzyme activity (Gardner et al., 1995; Yan et al., 1997). Protein damage owing to its oxidation has crucial role in disease pathogenesis. In Parkinson’s disease, a stretch of 20 amino acids at the C-terminus of $\alpha$-synuclein is oxidised (Glaser et al., 2005; Mirzaei et al., 2006). Oxidative inactivation of glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) downregulate glycolytic fluxes (Shenton and Grant., 2003; Hwang et al., 2009), oxidation mediated functional defects in mitochondrial protein is reported in amyotrophic lateral sclerosis (ALS) (Shi et al., 2010), cataract is known to occur in response to oxidative modification followed by cross linking of crystallin aggregates; the accumulation of cross linked high molecular weight aggregates is reported for lens opacity (Berthoud and Bever., 2009). Lysosome of aged and Alzheimer’s brain cells are found to have aggregates of peroxidised lipids and proteins called lipofuschin (Wolf et al., 1986), rheumatoid joint have oxidatively modified form of $\alpha$-1 protease inhibitor (Zhang et al., 1990; Janciauskiene and Strange., 2010). Oxidative
modification of protein generates autoreactive antibodies in diseases such as systemic lupus erythematosus (SLE), diabetes mellitus, RA, alcoholic liver disease etc. (Kurien and Scofield., 2008).

**Damage to lipids**

Lipid peroxidation can be defined as free radical mediated damage to polyunsaturated fatty acids (linoleic acid, arachidonic acid etc.) forming lipid peroxidation products. The reaction takes place during oxidative stress when the pro-oxidant/antioxidant balance shifts towards the former (Kurien and Scofield., 2007). Polyunsaturated fatty acids (PUFA) serve as excellent substrates for peroxidation reaction as they have active bisallylic methylene groups. The lower bond dissociation energy of C-H bond of the methylene group makes electron abstraction easier (Davies et al., 1981). The chance of lipid peroxidation increases with increase in the number of unsaturated bonds (Nagaoka et al., 1990). The process occurs in 3 phases: initiation, propagation and termination. In the initiation phase, the primary radical abstracts a H₂ atom from polyunsaturated fatty acid forming carbon centred fatty acyl radical. It combines with molecular oxygen in the propagation phase forming peroxyl radical and a chain reaction begins until the entire substrate is exhausted. Termination occurs when two fatty acyl radical combines to form lipid hydroperoxides (Kurien and Scofield., 2008). The process is self-sustaining and cause extensive damage to membranes both cellular and organelle. Besides this, enzyme catalysed lipid peroxidation is also reported. The enzymes involved are lipoxygenases and cyclooxygenases (Brash., 1999; Rouzer and Marnett., 2009). The damage decreases membrane fluidity and increase membrane leakage. The level of lipid peroxidation is measured either directly by measuring lipid peroxidation products (degradation products of lipid hydroperoxides) like malondialdehyde (MDA), hydrocarbons (ethane, ethane), α, β unsaturated aldehydes (4–HNE) or indirectly by decrease in the level of antioxidants enzymes like catalase, SOD, glutathione peroxidase etc or antioxidants like ascorbic acid, reduced glutathione, vitamin E. These degradation products are highly damaging and can form adducts with DNA and proteins bringing an alteration in its structure and this in turn is responsible for a variety of pathologies like cancer, neurodegeneration, atherosclerosis, aging etc (Steinberg et al., 1989; Ferreira et al., 1999; Marnett., 2000; Muller et al., 2007; Rahman., 2007; Grotto et al., 2009; Bradley et al., 2010).
Free radicals and diseases

Free radicals have an established role in the pathogenesis of over hundreds of human diseases directly or indirectly. The oxidative damage associated with ROS is involved in the progression of many diseases like atherosclerosis, carcinogenesis, myocardial reperfusion injury, amyotrophic lateral sclerosis (ALS) both familial and sporadic, liver damage, digestive system disorders, autoimmune nephrotic syndromes etc. (Liu et al., 1999; Waris and Ahsan., 2006; Ghodake et al., 2010; Jaescke and Ramachandran., 2011; Sugamura and Keaney., 2011; Braunersreuther and Jaquet., 2012; Kim et al., 2012). Free radical induces oxidation of low density lipoproteins (LDL) which are phagocytosed into sub-endothelial spaces forming foam cells. These cells damage endothelial cells causing atherosclerotic lesions. The lesions have deposits of cholesterol epoxides, lipid peroxides and autoantibodies for lipid peroxides (Stocker and Keaney., 2004). Free radicals have a prudent role in tumor formation, they promote transformation of normal cells to malignant ones (Sainz et al., 2012). ROS induces disorders of digestive system like gastric ulceration, hemorrhagic pancreatitis and inflammatory bowel disease (Jurjus et al., 2015). Build-up of toxic \(O_2^-\) occurs in ALS. The accumulation is due to defects is gene coding for Cu/Zn SOD on chromosome 21; thus causing a decline in SOD level which in turn results in oxidative stress (Julien., 2001; Roudeau et al., 2015). It has an established role in neurologic disorders like Parkinson’s disease where lipid peroxidation products, DNA/protein oxidation product are found in substantia nigra of Parkinson’s patients (Lotharius and Brundin., 2002; Dias et al., 2013). Markers of oxidative stess are also found in patients suffering from brain trauma, Alzheimer’s disease, Huntington’s disease, dementia and Downsyndrome (Benzi and Moretti., 1995; Bennett et al., 2009; Kovacic and Somanathan., 2012; Rodriguez-Rodriguez et al., 2014). Schizophrenic patients also have free radical overload, the breath of these patients have a typical pentane gas (a marker of lipid peroxidation) smell (Wu et al., 2013). Along with it, oxygen radicals have a role in alcoholic myopathy, malignant hyperthermia, alcohol induced liver heart and muscle disease, pulmonary edema, intraventricular haemorrhage, postasphygial CNS injury and eye diseases (cataract, degenerative retinal disease, ocular haemorrhage) also involve oxygen radicals (Reinke et al., 1987; Yagi., 1987; Halliwell and Gutteridge., 1988; Shaw., 1989; Arthur and Duthie., 1991; Bunce., 1992; Kehrer., 1993; Preedv et al., 2002). As most
unsaturated lipids are found in eye, they are highly vulnerable to oxidative damage (Houtsmuller et al., 1980). Reactive species of oxygen are associated with a wide range of autoimmune and inflammatory diseases like RA, glomerulonephritis, vasculitis, diabetes etc. (Gwinner and Grone., 2000; Mittal et al., 2014). In RA joint inflammation followed by infiltration of phagocytic cells occur at a high level. Accumulated neutrophils release oxygen radical into extracellular fluid which then damage macromolecular components (Stohs., 1995).

Antioxidants

The damaging effects of free radicals can be prevented by an array of antioxidant defences. The defence system has enzymatic and non-enzymatic antioxidants (Weydert and Cullen., 2010).

Enzymatic antioxidants can be categorised into:

(a) Superoxide dismutase
(b) Catalase
(c) Glutathione peroxidase
(d) Glutathione reductase
(c) Glutathione-S-transferase

(a) Superoxide dismutase (SOD)

SOD is a very important antioxidant enzymes that requires metal ions for their activation. The main function of SOD is to catalyse the conversion of O$_2^-$ into H$_2$O$_2$ and O$_2$ (Fukai and Ushio-Fukai., 2011). Mammals are known to have 3 isoforms in mammals namely :

*Cytoplasmic Cu/Zn SOD (SOD 1)*

*Mitochondrial Cu/Zn SOD (SOD 2)*

*Extracellular Cu/Zn SOD (SOD 3)*

Each of the enzyme is encoded by a different gene present in different sub-cellular location.

SOD1 is found mainly in cytosol with minor activity being detected in intermembranous space of mitochondria. It is encoded by SOD1 gene located on
chromosome 21 and requires Cu$^{2+}$ and Zn$^{2+}$ ions for disproportionation of O$_2^-$ to H$_2$O$_2$ and O$_2$ (Rosen et al., 1993; Milani et al., 2011; Estacio et al., 2015; Sea et al., 2015). The major site for SOD2 is the mitochondrial matrix where it is directed after synthesis in cytosol. It is encoded by SOD2 gene found on Chromosome 6. The disproportionation reaction is catalysed by Mn$^{2+}$ located at the active site of enzyme. Disruption in SOD2 activity is associated with neurodegeneration, cancer and aging (Pias et al., 2003; Perry et al., 2010; Becuwe et al., 2014; "Entrez Gene: SOD2 superoxide dismutase 2, mitochondrial"). SOD3 is localised in vascular extracellular matrix and cell surfaces. It is highly active in tissues as it binds them via its heparin binding domain (Faraci and Didion., 2004).

(b) Catalase

This enzyme scavenges H$_2$O$_2$ and dismutates it into H$_2$O and O$_2$ thus protecting cells from its toxicity (Sofo et al., 2015). The enzyme is localised in every cell and is highly active in H$_2$O$_2$ producing cells (Mostafa Abd El-Aal., 2012).

(c) Glutathione peroxidase (GPx)

It is a glycoprotein discovered by Mill in 1957 (Mills., 1957). The selenium dependent enzyme has selenocysteine at the active site of each of its subunit (Lubos et al., 2011). It is localised in cytosol and mitochondria where it catalyses the breakdown of H$_2$O$_2$ similar to catalase (Day., 2009; Ribas et al., 2014). GP$_X$ also oxidises reduced glutathione (GSH) to its oxidised form (GSSG). It has 5 isozymes each having varying sequence of amino acids, tissue distribution, location and substrate specificity (Dudek et al., 2002; Mostafa Abd El-Aal., 2012).

(d) Glutathione reductase (GSH)

The flavin containing enzyme is involved in glutathione metabolism converting GSSG to GSH in an NADPH dependent reaction, thus maintaining a high GSH/GSSG ratio (Meister, 1988; Dudek et al., 2002; Deponte., 2013).

(e) Glutathione-S-transferase (GST)

The enzyme provides defence against xenobiotics such as carcinogens, mutagens, anticancer drugs etc. by conjugating them with glutathione. It helps in reducing the reactivity of xenobiotics.
Non-enzymatic antioxidant

Apart from enzymes, a variety of non-enzymatic antioxidants like ascorbic acid (vitamin C), glutathione, phenolic compounds, vitamin A, vitamin E and β carotene are also found in human body. Non-enzymatic antioxidants can be categorized into:

(a) Vitamin E

The fat soluble vitamin is a family of compounds categorised into

Tocopherol (α,β,γ,δ)

Tocotrienol (α,β,γ,δ) (Brigelius-Flohe and Traber, 1999)

Amongst them, α tocopherol is most important having highest biological activity. It is abundantly found in safflower oils, wheat germ oil and sunflower (Reboul et al., 2006).

(b) Ascorbic acid

Ascorbic acid is one of the essential and important vitamins for maintaining human health. Due to its antioxidant properties, it is involved in many physiological functions in human body and is widely used in food industry as preservative. Besides antioxidant property, it also possesses anti-carcinogenic and anti-arthrogenic properties (Naidu., 2003).

(c) Carotenoids

They include a family of antioxidants notably lycopene, β-carotene, oxycarotenoids and their main action is quenching of free radicals (Sies et al., 1992; Rahman., 2007). Thus, they help in protecting biological macromolecules (DNA, proteins) from damaging effects of free radicals to some extent. This in turn reduces the incidence of a variety of ROS related diseases.

Methylglyoxal (MG)

The research on MG started in the last decade of 19th century and it was at that time when synthesis, chemical characterisation and reactions of MG started (Baumann., 1885; von Pechmann., 1887). MG, a highly reactive endogenous α-oxoaldehyde is a deceptively small molecule formed during the metabolism of biological macromolecules namely glucose, fatty acids and protein (Desai et al., 2003).
Introduction

2010). It is present in unhydrated, monohydrated and dehydrated forms in aqueous solution with monohydrate being most abundant (71 %) of all (Creighton et al., 1988; Thornalley., 1996; Rabbani and Thornalley., 2014). This yellow coloured liquid with pungent odour has 20,000 times more reactivity than glucose (Thornalley., 2005; Vistoli et al., 2013). It has a potential role in disease development by modifying biological macromolecules. Its concentration in body fluids can be determined by derivatization with 1, 2-diamino-4,5-dimethoxybenzene or HPLC of the resulting quinoxaline adduct and also by UV or fluorescence (McLellan et al., 1992). Whole blood samples of healthy human subjects contain 80 nm of MG. Relatively high concentration (1-2 μM) are found in lens (Haik et al., 1994; McLellan et al., 1994) (Fig. 10).

![Structure of methylglyoxal](www.sigmaaldrich.com)

**Fig. 10:** Structure of methylglyoxal

**Source** www.sigmaaldrich.com

**Sources of MG**

MG can be obtained from numerous sources. They can be classified into exogenous and endogenous sources.
Exogenous sources

It is a potential environmental hazard present in almost all food items, main sources being mono-, oligo-, polysaccharides and lipids (Degen et al., 2012). Several processes like auto-oxidation, photodegradation, heating and prolonged storage of food items lead to its generation. Retroaldol condensation and auto oxidative fragmentation of sugars generate MG. Coffee, cigarette smoke, alcoholic drinks represent good sources of this toxic dicarbonyl (Casazza et al., 1984; Fujioka and Shibamoto., 2006; Wang and Chang., 2010). It is also found in yoghurt, baked cookies, soya sauce and honey (Nagao et al., 1986; Adams et al., 2009; Kuntz et al., 2009; Kocadagıt et al., 2016). Micro-organisms increase MG content in alcoholic drinks and fermented foods (Cooper., 1984). Urban atmosphere is an important source of MG. It is also generated by combustion in cigarette smoke and in automobile exhausts where hydrocarbon, toluene compounds, other aromatic compounds undergo incomplete combustion. Besides oxidation of many volatile organic compounds give rise to this dicarbonyl compound. Isoprene produces 79 % of MG globally with acetone being the second largest source of MG giving 7 % of total MG in atmosphere annually (Fu et al., 2008). It is also produced on oxidation of monoterpenes by ozone (Yu et al., 1998; Fick et al., 2003; Nunes et al., 2005). Pinene, a monoterpane contributes 1-11 % of total MG globally (Fick et al., 2003; Fick et al., 2004). The environmental MG is fixed into the soil by rain water, fog and mist (Fu et al., 2008). Drinking water also gets contaminated by this toxic dicarbonyl during purification process mainly ozonation and chlorination (Camel and Bermond., 1998; Fu et al., 2008).

Endogenous sources

It is a biological metabolite having numerous in vivo origins. It is formed inside the cell mainly from triose phosphate intermediate of glucose metabolism but a minor fraction leaks (Phillips and Thornalley., 1993). Endogenously, it can be formed both enzymatically and non-enzymatically.

Enzymatic sources

The enzymes involved in catalysing the formation of MG include MG synthase, cytochrome P450 2E1 and semicarbazide sensitive amine oxidase (SSAO)
Introduction

(Murata et al., 1985; Angeloni et al., 2014). The enzyme participates in glycolytic bypass catalysing conversion of triose phosphate to dihydroxyacetone phosphate (DHAP) (Cooper., 1975; Cooper., 1984). Cytochrome P450 2E1 participates in acetone metabolism. The conversion requires two consecutive steps via acetal as intermediate (Casazza et al., 1984; Koop and Casazza., 1985). Other important compounds that give MG via acetal intermediate are ketone bodies, β-hydroxyl butyric acid and acetoacetic acid (Beisswenger et al., 2005). SSAO an amine oxidase named because of its sensitivity to semicarbazide inhibition catalyses aminoacetone to MG conversion (Elliott., 1960; Urata and Granick., 1963; Lyles., 1996). The pathway is exacerbated in low CoA states (Callingham et al., 1995; Lyles., 1996). Threonine and glycine catabolism generates MG via aminoacetone (Deng and Yu., 1999). Both soluble and tissue bound form of SSAO are potent MG producers (Lyles., 1996). It is also produced from glycerol by enzyme glycerolphosphatase.

Non-Enzymatic sources

Mainly spontaneous degradation of triose phosphate intermediate to glyceraldehyde-3-phosphate (G-3-P) and dihydroxyacetone phosphate (DHAP) produces MG (Sato et al., 1980). Besides auto-oxidation of sugars (Hayashi et al., 1986; Keyhani and Yaylayan., 1996), lipids and proteins are other important non enzymatic MG producing sources (Wolff and Dean., 1988; Jiang et al., 1990) (Fig. 11).

Degradation of MG

The main MG degrading system is the glyoxylase system and it provides first line of defense against MG induced toxicity (Fig. 12). The system comprises of two enzymes glyoxylase I, a metalloprotein and glyoxylase II, a metallohydrolase as well as catalytic amount of reduced glutathione (Racker., 1951). Glutathione catalyses the conversion of MG into hemithioacetal that acts as a substrate for glyoxylase I which converts it into S-lactoyl glutathione, a substrate for glyoxylase II. It is finally degraded to D-lactate (Vander Jagt., 1989; Mannervik and Ridderstrom., 1993). According to estimates only 0.3 % of the total MG produced/day reacts and forms glycated adducts, rest is metabolised by glyoxylase I (Rabbani and Thornalley., 2012). Other enzymes beside glyoxylase system involved in MG detoxification are α-
oxoaldehyde dehydrogenase, betaine aldehyde dehydrogenase, aldose reductase and 2-oxoaldehyde. 2-oxoaldehyde is an important liver detoxification enzyme that require vicinal aminoalcohol as cofactor (Dunkerton and James., 1975; Dunkerton and James., 1976; Vander Jagt and Davidson., 1977; Vander Jagt., 1982). Aldehyde dehydrogenase has 17 or more functional gene, 3 of these perform detoxification of aldehyde (Hempel and Jornvall., 1989). MG is 104 times better substrate for aldose reductase than glucose (Vander Jagt et al., 1992).

Fig. 11: Mehanistic interpretation of glyoxal, methylglyoxal and 3-deoxyglucosone formation in early glycation

Source Thornalley et al., 1999
Fig. 12: Major detoxification pathways of MG

Source Rabbani et al., 2016
MG role in glycoxidation

MG is an intermediate of maillard reaction and is a potent glycating agent showing 20,000 times more reactivity than glucose (Thornalley., 2005). Literature suggests that glycation by MG is linked to oxidation i.e. glycation and oxidation are mutually interlinked in vivo. First report of ROS generating ability of MG in cellular systems appeared in 1993 (Kalapos et al., 1993). Free radicals are produced both during formation and degradation of MG. During the process of protein modification by MG reactive radical producing centres are formed (Lee et al., 1998; Ortwerth et al., 1998; Dornadula et al., 2015), eg MG modified complex III of mitochondria has been shown to generate enhanced $O_2^-$ (Rosca et al., 2005) and this in turn damage mitochondria. ROS producing NADPH oxidase is activated when AGES are formed upon reaction of MG with biological macromolecules bind to their receptors (Wautier et al., 2001; Yan et al., 2003; Tan et al., 2007). MG also impairs ROS detoxifying enzymes like glutathione reductase, superoxide dismutase, glutathione peroxidase again leading to in vivo oxidative stress. It inactivates Cu-Zn SOD by covalent crosslinking to the enzyme, releasing Cu$^{2+}$ ions (Ortwerth et al., 1998). In blood cells MG has been found to interfere with antioxidant enzymes (Beard et al., 2003; Nicolay et al., 2006). Thus, free radicals are concomitantly produced by AGE or AGE-receptor interaction (Sakurai and Tsuchiya., 1988; Mullarkey et al., 1990; Yan et al., 1994; Lander et al., 1997). Free radicals are also produced from reactive centers generated by crosslinked proteins. These centers catalyse one electron oxidation reduction with appropriate substrates. It has been reported that 100 mmol/l of MG increased NADPH oxidase mediated $O_2^-$ production in rat mesangial cells (Ho et al., 2007). It increase oxidative stress in cultured neural cells from rat hippocampus through induction of IL-1β and IL-6 cytokines. It increased ROS formation and induced apoptosis of cultured cortical neurons (Kikuchi et al., 1999; Shinpo et al., 2000). Free radicals are also generated during MG formation from aminoacetone or acetol. Auto-oxidation of aminoacetone to MG mediated by metal ions such as Fe$^{2+}$ and Cu$^{2+}$ is considered a source of carbon centered radicals and superoxide (Hiraku et al., 1999; Dutra et al., 2001). A wide range of cellular system e.g. vascular smooth muscle cells, rat hepatocytes, neurons, platelets, endothelial cells have shown to produce ROS in relation to MG (Angeloni et al., 2014). It is the most reactive endogenous carbonyl compound able to induce AGES which in turn stimulates
production of cytokines and growth factors (Lo et al., 1994; Westwood and Thornalley., 1996; Oya et al., 1999; Wautier et al., 2001; Basta et al., 2002; Chen et al., 2002; Kikuchi et al., 2003). It plays an important role in Alzheimer’s disease progression acting as a neurotoxic mediator of oxidative stress activating many redox signalling pathways ultimately leading to apoptosis and cellular dysfunction. Two other kind of free radicals, cross linked radical cation and MG radical anion are generated in MG system along with ROS (Yim et al., 1995).

MG damage to biological macromolecules

MG is a reactive electrophile that reduces the biological activity of various macromolecules by attacking them (Fig. 13). The nature of macromolecule determines the consequence of modification of macromolecular motif. Proteins (side chain of arginine and lysine residues), amino group of nucleic acid and basic phospholipids can be targeted by MG (Silva et al., 2013). It reacts rapidly with proteins (Riley and Harding., 1995) almost exclusively with arginine and to a lesser extent with lysine, cysteine and tryptophan residues. Arginine residues with functional domains are hot spots for MG modification eg. Arginine residues within RGD and GEOGER motifs of integrin binding domain of collagen IV lining the wall of blood vessels are hot spots for MG glycation (Dobler et al., 2006; Angeloni et al., 2014). Cyclic imidazole adduct (MG-H) and other related structural isomers [Nδ-(5-hydro-5-methyl-4-imidazolon-1-yl)] (MG-H1), [2-amino-5 (2-amino-5-hydroxy-5-methyl-4-imidazolone-1-yl) pentoic acid] (MG-H2) and [2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolone-1-yl) pentanoic acid] (MG-H3) are formed as a result of MG modification by arginine (Frye et al., 1998; Thornalley et al., 2003). Further reactions by these molecules yield [Nδ-(4-carboxy-4, 6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydro-pyrimidine-2-yl) L-ornithine] THP or argpyrimidine. With lysine residues it gives [Nε (1-carboxyethyl) L-lysine (CEL) and [Nε (1- carboxy methyl) L-lysine] (CML) adducts and the lysine dimer [1,3-di (Nε-lysine)-4-methyl imidazolium] (MOLD). The compound formed on MG interaction with one arginine and one lysine residue is called methylglyoxal-derived imidazolium cross-link (MODIC) (Shipanova et al., 1997; Oya et al., 1999; Bieme et al., 2002; Ahmed et al., 2003) (Fig. 14). It reacts with cysteine and tryptophan forming reversible hemithioacetal adduct and β carboline derivative respectively (Lo et al., 1994; Nemet and Defterdarovic., 2007).
Along with it EPR spectroscopy results of MG reaction with BSA and casein suggested unidentified protein free radical formation (Gascoyne., 1980; Mc Laughlin et al., 1980). MG mediated glycation can potentially damage sensitive areas in blood vessels and tissues. MG modified mitochondrial protein and LDL can become a source of oxidative stress and atherosclerotic plaque formations (Thornalley and Rabbani., 2011). The accumulated AGE also causes damage to lipids, DNA, RNA, proteins and glycoconjugates (Baynes., 2000; Baynes., 2001). MG can also irreversibly modify DNA forming nucleotide AGEs, the most reactive target being deoxyguanosine and so MGdG is the most common nucleotide AGE formed. In vitro cultured human and bovine cells have shown glycated DNA in them (Schneider et al., 2006; Thornalley et al., 2010; Mustafa et al., 2012). Loss in genomic integrity and intra strand crosslinks were detected in glycated DNA. These interactions are shown to have carcinogenic, mutagenic and teratogenic effects. It has been reported that chinese hamster ovary cells on exposure to MG forms cross-linked protein-DNA adducts (Brambilla et al., 1985). Onset of cardiovascular, neurological, connective tissue disorders as well as aging is found to be associated with DNA damage. Lipid linked AGEs are formed on MG-lipid interactions. A lipid linked AGE, carboxymethylethanolamine is found in vivo and is a biomarker of lipid glycation during maillard process. Phospholipids can readily react with other dicarbonyl compounds besides MG. Streptozotocin induced diabetic rat liver shown high content of glycated phospholipids (Bucala et al., 1993; Pamplona et al., 1995; Requena et al., 1997a; Brown et al., 2005; Desai et al., 2010) (Fig. 15).
Fig. 13: Methylglyoxal provoked toxicity
Source Kalapos., 2008

Fig. 14: Major pathways for the formation of the methylglyoxal-derived AGEs in vivo
Source Vistoli et al., 2013
**Introduction**

Several routes are known for methylglyoxal formation in living cells, although its main production pathway is associated to glycolysis, either non-enzymatically or through the action of methylglyoxal synthase (found only in bacteria until recently). Once formed, it readily forms adducts with thiol (glutathione, trypanothione and cysteine residues in proteins) and amino (proteins, nucleic acids and lipids) groups. Irreversible reactions with amino groups in proteins and nucleic acids lead to MAGE formation and functional impairment of these macromolecules. Coupling an irreversible enzymatic pathway that eliminates methylglyoxal, the glyoxalase system, greatly reduces glycation. The main known role of this pathway is thus as an effective glycation preventor.

**Source**  Silva et al., 2013

**MG in relation to diabetes**

MG plays a very important role in diabetes and its long term complications. It affects insulin secretion by β cells of pancreas, reduces the activity of β cells and so secretion of insulin and can also induce swelling and apoptosis. Moreover it is known
to bind to insulin chain and form adduct. Clearance of MG-insulin adduct by kidney as well as signaling pathways that require insulin are impaired (Jia et al., 2006; Pi et al., 2007). During diabetes, MG mediated AGE formation is elevated. These AGES are a source of various age related, neurodegenerative and cardiovascular disorders. A positive correlation is found between plasma protein modified by MG and glycohaemoglobin (Uchida et al., 1997; Shamsi et al., 1998). There are multiple pathways of MG formation and degradation. MG sources are accentuated in diabetes. Plasma lactate concentration increases; glucose proteolysis leads to increased acetone level which in turn establishes ketotic state. Plasma level of MG is found to increase up to 3.26 ± 0.7 mM in ketotic state. Along with it activity of MG forming enzyme CYP2E1 increases two to four fold during diabetes (Bellward et al., 1988; Barnett et al., 1994). The activity of MG degrading glyoxylase system is also found to be diminished. The enzyme require GSH to convert MG to D-Lactate. Activity of GSH is diminished as a consequence of increased oxidative stress during disease progression and so it leads to glyoxylase decline (Haik et al., 1994; Tiwari et al., 2013; Allaman et al., 2015) (Fig. 16).

Fig. 16: Methylglyoxal and diabetes
Source Rabbani and Thornalley., 2014
Introduction

Role of MG in other diseases

Besides diabetes, other diseases involving glycation of protein, increased protein breakdown, protein deposits have a direct relation with MG concentration. MG toxicity is related to ROS production ultimately ending in necrosis or apoptosis of cell. It induces tumor. Beri-Beri is suggested to be linked to MG overproduction. It also has a role in Alzheimer’s disease and inborn errors of metabolism (Vogt-moller., 1929; Salem., 1954; Kalapos., 2008; Angeloni et al., 2014).

Functions of MG

1. In yeast the compound acts as a signal initiator activating the osmo sensor sln1, HOG-MAP kinase pathway and calcium signaling pathway (Thornalley., 1996; Creighton et al., 2003).

2. It has a physiological role in modulation between aerobic and anaerobic bioenergetics (Bento et al., 2010).

3. It has an important role in signaling pathways.

4. Anxiety supressing effects of MG are found in mice brain (Hambsch et al., 2010).

5. It has pathophysiological roles, receptors on macrophages are found to target and endocytose albumin modified by MG (Westwood et al., 1994).

6. It acts as a neurotoxic mediator in Alzheimer’s disease activating signalling pathways leading to dysfunction and/or apoptosis of cell (Angeloni et al., 2014).

7. It plays an important role in signal transduction, cell energetics, maintaining redox status and controlling functions of cell (Angeloni et al., 2014).

8. Impaired detoxification and increased production of MG can cause cognitive decline in Alzheimer’s disease (Krautwald and Munch., 2010).

9. In Alzheimer’s disease it increases hyperphosphorylation of tau protein by regulating kinase and phosphatase levels (Gong., 1994; Wang et al., 1995; Planel et al., 2004; Hu et al., 2008; Iqbal et al., 2009).
Toxicity of MG

There are numerous ways by which MG can exert its toxicity. Cells show different responses towards MG handling depending on the biochemical machinery of the cell. There are 3 main ways by which MG exerts its toxicity:

1. It can exert a direct inhibitory effect on enzymes, this occurs in early phase of toxicity called carbonyl stress. Glycolytic enzymes, intra mitochondrial enzymes, enzymes participating in cell defence, Na\textsuperscript{+}-K\textsuperscript{+} ATPase, and some transport proteins are shown to be inhibited by MG (Kun., 1950; Leoncini et al., 1980; Mira et al., 1991; Ferguson et al., 1998).

2. It can induce free radical production, which cause cellular necrosis or apoptosis. These cells are prone to further attack and so generates more of free radicals, thus creating a vicious circle. MG can also deplete glutathione stores.

3. The late phase of MG toxicity has genotoxic and carcinogenic effects. Besides these, the following toxic effects have also been reported:
   a) Pulmonary hyperemia and edema with degenerations of kidney, liver and intestine are associated with lethal doses of this dicarbonyl compound (Sjolemma and Seekles., 1926).
   b) Decline in the body weight within 4 hrs was found in mice injected with lethal doses of MG, however a slow decrease in weight occurred with reduced dose of the compound (Kalapos et al., 1991).
   c) Thickening of basement membrane of kidney glomeruli occurred as a result of oral administration of MG in female mice (Osterby., 1986; Golej et al., 1998).
   d) Specific activities of SOD, glutathione-S-transferase, glyoxylase I and II, catalase were found to be diminished on MG administration (Choudhary et al., 1997).
   e) Increase in MDA was also found in liver and spleen of animals as a result of MG administration (Choudhary et al., 1997).
   f) It is cytotoxic as it is reported to induce fragmentation and formation of apoptotic bodies when added to different cell lines (Okado et al., 1996).
Glycoxidation

Glycoxidation is a collective term used for two interrelated post translational modifications of great importance i.e. glycation and oxidation. The two are highly connected processes showing strict interdependency upon each other, thus forms the basis of molecular changes causing dysfunction of cell and alteration of tissue (Kristal and Yu., 1992; Guedes et al., 2011). In 1912, Louis Camille first discovered glycation reaction and so the process is now termed as maillard reaction (Maillard., 1912). Glycation is a process where the electronegative oxygen of carbonyl group (C=O) of sugars react non-enzymatically with electropositive amino group of biological macromolecules like DNA, proteins, lipids, to form Schiff base. Being unstable, it undergoes series of rearrangements, dehydration, cyclisation and then stabilizes to amadori product. These are called early glycation products (EGPs) and are in a state of equilibrium with their precursors (Brownlee et al., 1988). These EGPs have two fates i.e. they can either degrade and form reactive dicarbonyl compounds or can proceed to form highly stable advanced glycation end products (AGEs). These reactive dicarbonyls too end up with AGEs (Ahmad et al., 2014a). Pentosidine and CML are amongst the most common AGEs. It is at the amadori to AGE step that free radicals/ ROS are needed (Fu et al., 1992). Free radicals accumulate from a number of sources, most important being schiff base and amadori products themselves (Ahmad et al., 2011a; Akhter et al., 2013). Besides, a number of processes/agents/pathways under hyperglycemic conditions release free radicals. These include mitochondrial respiration, NADPH oxidase, xanthine oxidase, sorbitol pathway, PKC pathway (Bandeira et al., 2013). Glucose along with other hydroxyaldehydes too can undergo auto-oxidation via enediol intermediate, releasing H$_2$O$_2$, which can further transform to most reactive OH$^-$. Most of the free radical releasing mechanisms are accelerated in the presence of transition metal ions and inhibited in the presence of reducing compounds like ascorbate (Thornalley et al., 1984; Wolff and Dean, 1987; Baynes., 1991). Due to the prudent role of oxygen radicals in AGE formation, advanced glycation end products are now called advanced glycoxidation products (O’Brien and Timmins., 1994). Thus, it can be concluded that glycation acts through oxidative mechanisms and the two are related at the molecular level (Traverso et al., 1997; Islam et al., 2017).
Glycoxidative damage to proteins

Both intracellular and extracellular proteins can undergo non-enzymatic post translational chemical modifications that affect their function severely (Stadtman., 1990; Han and Martinage., 1992). Amongst them, two interrelated processes, glycation and oxidation are of special consideration which ultimately form advanced glycoxidation products (Mir and Moinuddin., 2015). Glycation is an inevitable non-enzymatic reaction that occurs between carbonyl group of reducing sugar like glucose, fructose, mannose or their phosphate derivatives and other related compounds such as reactive dicarbonyl compounds (glyoxal, methylglyoxal, glucosone, 3-deoxyglucosone), ascorbic acid etc and the electropositive amino (-NH$_2$) group of protein, mainly the ε-amino group of lysine forming unstable Schiff base and benign amadori adducts (Singh et al., 2001). This early stage of reaction is called non-enzymatic glycosylation or more properly glycation (Roth., 1983). These amadori products can either degrade to highly reactive dicarbonyl species or after a series of adjustments end up with a range of heterogenous, irreversible pigmented fluorescent compounds called maillard products or AGEs (Thornalley et al., 1999; Hansen., 2002). It is at the stage of amadori, that O$_2$– is produced and the process both of O$_2$– formation and its dismutation to H$_2$O$_2$, accelerates many fold in the presence of transition metal ions. These transition metal ions also accelerate free radical release from glucose auto-oxidation. Their important role is confirmed by a study conducted on BSA, where metal chelating agents (EDTA, DETAPAC) inhibits glucose induced conformational changes (Wolff et al., 1991). These ROS can cause significant damage to proteins along with glycative pathway. The two are so mutually dependent, that their individual existence cannot be thought. The glycoxidative damage causes structural changes and alter characteristic properties of both circulating and tissue proteins (Kennedy and Baynes., 1984; Baynes., 1991). It influences the spatial orientation of amino acids, block critical amino groups, induces protein carbonyl formation, provokes fragmentation, crosslinking (dityrosine), aggregation, loss of surface charge, increased susceptibility to proteolytic enzymes, formation of oxidative adducts like CML and pentosidine (Yamada et al., 2004; Pashikanti et al., 2011). The modified protein further cause secondary damage on other biomolecules like enzymes, NADPH and lipids thus causing a variety of pathologies (Pongor et al., 1984; Harding., 1985; Cerami et al., 1986; Breitling-Utzmann et al., 2001; Baynes.,
A wide variety of proteins and enzymes of clinical significance have been affected by glycation. The structural alteration in histone post glycation causes secondary and tertiary protein modifications that generate neoepitopes capable of causing aggressive immune response (Mir and Moinuddin., 2015). Albumin on modification shows diminished ligand binding capacity (Shaklai et al., 1984). Besides, proteins involved in a variety of cellular processes like bioenergetics, glucose metabolism and cell repair loses their functional capability post glycation (Ahmad et al., 2014a). Glycation of hemoglobin is of marked importance in diabetes. Lipoprotein glycation promotes its binding to vessel wall enhancing chances of atherosclerosis (Lyons., 1993). A decline in thrombotic tendency occurs post anti-thrombin III glycation (Lyons., 1993). Collagen being most abundant in the body is highly exposed to glycoxidative damage leading to arterial stiffness and altered flow dynamics (Lyons., 1993), fibrinogen glycation affects fibrin network kinetics declining lysis rate of fibrin clots (Pieters et al., 2008). A decline in iron binding capacity of transferrin is reported post glycation (Van Campenhout et al., 2003). Besides numerous other proteins and enzymes like human complement regulatory protein (HCD 59), creatine kinase, amino transferase, glucose metabolizing enzymes mainly glyceraldehydes-3-phosphate dehydrogenase, pancreatic glucokinase are either inactivated or undergo activity loss post glycation (Zhao et al., 2000; Beranek et al., 2001; Qin et al., 2004).

**Glycoxidative damage to DNA**

Similar to proteins DNA also forms advanced glycoxidation products. Glycoxidative damage to DNA alters its structure that include unwinding of double helix, depurination, strand breaks, mutations (insertion, deletion, transposition). The structural alteration further causes change in function and has genotoxic effects (Ahmad et al., 2011b; Mustafa et al., 2012; Ahmad et al., 2014b).

**Glycoxidation markers**

AGEs are highly stable and accumulate in a variety of pathologies thus serving as marker for monitoring disease status. CML and its related products, pentosidine are amongst the most common AGEs formed by combined action of glycation and oxidation. Its level is reported to be elevated in many pathologies (Hodge., 1953;

**Glycoxidation and diseases**


**Diabetes and glycoxidation**

Evidences suggest that advanced glycation and oxidation of structural proteins accelerates in diabetes. Elevation in blood glucose exposes macromolecules to glycoxidative damage, thus resulting in structural and functional perturbations. The serum and tissue AGE level correlate with the degree of complications (Monnier *et al.*, 1986; Makita *et al.*, 1991; Brownlee., 1992). AGEs also promote diabetes by causing resistance to insulin and stimulating low grade inflammation (Tahara *et al.*, 2012). The oxidative chemistry of glycation also plays an important role in diabetes associated tissue damage (Wolff., 1987). A number of processes, as described above generate ROS during glycation. Also antioxidant defenses both enzymatic (catalase, SOD) and non-enzymatic (ascorbic acid, vitamin E, uric acid, glutathione) are compromised, further contributing to oxidative stress (Herman *et al.*, 1976; Matkovics *et al.*, 1982). AGEs are found to accumulate more in long lived proteins like collagen making it brown and fluorescent. The loss in tensile properties causes arterial stiffness, loss of elasticity and a decline in lung capacity (Pillsbury *et al.*, 1974; Grgic *et al.*, 1975). Glycoxidation of circulating lipoprotein and lens protein causes atherosclerosis and cataract in diabetes (O’Brien and Timmins., 1994).
RA and glycoxidation

Raised AGE level are also found in the serum and synovial fluid of RA patients. It is the oxidative stress during local and systemic inflammation that accelerates AGEs formation and plays important role in acute and chronic complications of diabetes (Maurice et al., 1999). Pentosidine and CML, the well characterized AGEs and markers of oxidative stress are found raised in articular cartilage as well as serum and synovial fluid of RA patients (Takahashi et al., 1994; Miyata et al., 1998). Drinda et al., reported CML in synovial lining, sublining, stroma cells and endothelium of synovial vessels in his experiments (Drinda et al., 2002).

Diabetes

Diabetes mellitus is the most common non communicable endocrine disorder of high global prevalence. It is a greek word, diabetes meaning “passing through a siphon” and mellitus means “sweet”. They named it when they observed the urine of such patients attracted flies and bees. The traditional way of diagnosing diabetes by ancient chinese is by observing if ants are attracted to the urine of such patients (Patlak., 2002). `It is the fifth largest cause of death arising from defects in insulin secretion or its action. The insulin defects cause disturbance in storage and mobilisation of fuels leading to improper handling of carbohydrate, fats and proteins (Zimmet., 2000; Kumar and Clark., 2002; Beverley and Eschwege., 2003). The prolonged disturbances cause complications like retinopathy, neuropathy, nephropathy, cardiovascular complications, ulceration, organ failure with accompanying peripheral vascular and cerebrovascular diseases (Liu et al., 2010). Due to widespread prevalence and severe complications, the disease must undergo extensive research to find out economical and better solutions.

History

The history of diabetes mellitus dates long back, and the disease is known since ages. It is in middle ages that treatment of diabetes started followed by elucidation of its pathogenesis in 20\textsuperscript{th} century (Patlak., 2002). In 1889, Joseph Von Mering and Oskar Minkowski discovered pancreas as the major organ involved in diabetes. They observed removal of pancreas from dogs caused diabetes, and injecting them with extract from pancreatic islet lead to reversal of symptoms. This extract was
later named insulin (Himsworth., 1936; Patlak., 2002). With the discovery of insulin, large scale isolation from bovine pancreas started at Toronto University, Canada. It was effectively used to treat patients with first clinical trial in 1922. With the passage of time other landmark discoveries like distinction between type I and type II diabetes, identification of effective drugs like sulphonylureas and thiazolidinediones took place (Himsworth., 1936; Patlak., 2002).

**Epidemiology**

It is a disorder of glucose intolerance that is increasing to epidemic proportions throughout the world and ranks 5th in causing death worldwide (Roglic et al., 2005; Tabish., 2007). According to estimates, 285 million people aged between 20 to 79 suffered from diabetes in 2010. Of all, 70 % were from developed countries; 40 million of the total were from India and the number is expected to increase to 300 million by 2025 and 438 million by 2030 (Sicree et al., 2006; Piero et al., 2010). The disease affects almost every population of the world. Approximately 7 % of the world population and 5-7 % population globally is facing the disease (WHO., 1994; Amos et al., 2010; King et al., 1998; Harris et al., 1998). As per WHO reporting, African region is more prone to diabetes. An estimated 7.02 million people were diabetic in 2000, out of this only 0.1 % are type 1 diabetic with rest type 2 (WHO., 2008; Kirigia et al., 2009). The latter is predominant form of diabetes accounting for 90 % of all diabetes cases. Due to its strong correlation with the genetics of an individual, the prevalence varies with < 1 % in certain populations to over 50 % in Pima Indians of Arizona (Zimmet., 1982). Besides, life style is also a major contributing factor in its incidence. Western culture, over-weight, obesity all promote the disease making it the 5th leading cause of death worldwide. Serious concern, awareness, modulation of lifestyle, effective drugs, active reaseach and a lot more is needed to be done to combat increasing statistics of the disease (Fig. 17).
Introduction

Fig. 17: A time and habitat dependent rise in diabetes

Diagnostic criteria of diabetes

It is World Health Organisation (WHO), National Diabetes Data Group (NDDG), and American Diabetic Association (ADA) that put forward the disease classification in different era’s. WHO first classified the disease in 1965 followed by time to time modifications in criterion. In 1979, NDDG modified the 1965 diabetic criterion. Changes were made further by WHO in 1985, ADA in 1997. The latest diagnostic criteria were put forward by WHO in 1999. According to the criteria, a person is considered diabetic if:

1. He/she exhibit symptoms of the disease like polyurea, polydipsia, unexplained weight loss.
2. Fasting Plasma Glucose (FPG) = 7 mmol/L.
3. 2hr Plasma Glucose = 11.1 mmol/L.
4. HbA1C > 6.5 % provided the testing laboratory should be NGSP certified or DCCT assay standardised (NDDG, 1979; WHO, 1980; WHO, 1985).

Causes

The leading cause is defective insulin secretion or action. The defects are mainly due to destruction of pancreatic β cells (insulin producing cells) or mutations in insulin receptor that limits insulin action (Raffel et al., 1997). Any disturbance in
the action of insulin may it be excess production of insulin counter regulatory hormones as occurs in Cushing’s syndrome, acromegaly may result in diabetes (ADA., 2001). It could also be an outcome of side effects caused by certain drugs like glucocorticoids, pentamidine, niacin, α- interferon etc. (Pandit et al., 1993).

Classification

The classification criteria are in accordance to etiology and clinical presentation. The disease is established by expert committee of WHO in cooperation with NDDG. It has 4 broad categories (Sicree et al., 2006).

Type 1 diabetes mellitus (T1DM)

Also called insulin dependent diabetes (IDDM) as the patients lack insulin completely. The possible reason may be immune destruction of pancreatic β cells or idiopathic. As most of the cases are reported in early age (< 20 years), it is also called juvenile onset diabetes mellitus.

Type 2 diabetes mellitus (T2DM)

It is the major cause of diabetes and is also called non-insulin dependent diabetes mellitus (NIDDM). The patients have decreased sensitivity of insulin to target tissues. It affects people after 40 years of age, so is called maturity onset diabetes. The difference between type 1 and type 2 diabetes mellitus is shown in figure 18.

Gestational diabetes mellitus (GDM)

Gestational diabetes is a state of high blood sugar level that develops during pregnancy and generally disappears after giving birth.

Other specific types

Diabetes that occurs as a result of any of the following are categorised into other specific types.

It includes:

- Genetic defects in β cell function
An Introduction

- Genetic defects in insulin action
- Side effect of drugs or chemicals
- Disease of exocrine pancreas
- Infections
- Endocrinopathies (ADA., 2009)

Fig. 18: Difference between type 1 and type 2 diabetes

Source Forbes and Cooper., 2013

Type 1 diabetes mellitus (T1DM)

Type 1 diabetes accounts for 5-10% of all the cases (Daneman., 2006). Thus minority of total burden of diabetes goes to this type. Although it occurs in all age groups but the majority of cases are reported in children (Amutha et al., 2013). Owing to its higher incidence in western society, it is called “disease of wealth” (Patterson et al., 2001). According to epidemics, there is a 3.4% increase in incidence of type 1 diabetes in children under 15 years of age, most of which are below 5 years. The rise is seen across europe. People of Asian and African origin are more prone to
the disease (Ahren and Corrigan., 1984; EURODIAB ACE., 2000). It is further classified into 2 types:

**Type 1a**

There is greater incidence of type 1a diabetes with 90% cases seen in europe. The disease is characterised by immunological destruction of pancreatic β cells causing little or no insulin secretion. Defective insulin production by β cells of islets of Langerhans leads to impaired glucose metabolism causing persistent hyperglycemia (Bastaki., 2005; Raju and Raju., 2010). Many autoimmune diseases like Graves’ disease, autoimmune thyroiditis, Addison’s disease are associated with this type of diabetes. Such patients also have abundant autoantibodies like islet cell cytoplasmic antibodies (ICA), islet cell surface antibodies (ICSA), anti-insulin antibodies (IA), anti-glutamic acid decaboxylase antibodies (anti-GAD). ICA and ICSA are found in 90 and 80% of all type 1a diabetes cases and are directed against islet cell cytoplasmic and surface proteins respectively (Betterle et al., 1983; Atkinson and Maclaren., 1994; Zimmet et al., 2004; Raju and Raju., 2010).

**Type 1b**

It has idiopathic reasons with no known etiological basis. The disease accounts for 10% of all type 1 diabetes cases. Some patients report permanent insulinopenia and ketoacidosis (Ahren and Corrigan., 1984). A range of metabolic derangements come into action as a consequence of the disease progression. It is not only the destruction of pancreatic β cells leading to insulin deficiency but also abnormal action of pancreatic α cells, causing excessive production of glucagon (a counter regulatory hormone). Besides, target tissue response to insulin also declines. Glucose no longer serves as fuel source and the cells rely on alternative sources causing abnormality in protein, fat and lipid metabolism (Jiang and Zhang., 2003). Besides, polyuria, polydipsia, ketoacidosis also occurs and chronic hyperglycemia affect other organs of the body (Perilli et al., 2013). A decline in the function of associated genes like glucokinase in liver and GLUT 4 in adipose tissue also occurs due to defects in glucose transport (Raju and Raju., 2010; Ozougwu et al., 2013).
Type 2 diabetes mellitus (T2DM)

It is a very common non communicable, non-autoimmune disease. Increased morbidity and associated mortality make it a life-long disorder. It has a high prevalence among all types of diabetes accounting for 85–95% of all diabetic cases. It is more prevalent in developing countries in comparison to developed nations (Narayan et al., 2006; Tabish., 2007; Deshpande et al., 2008; Al-Daghri et al., 2011). Disorders associated with secretion or action of insulin is the established cause of diabetes. Due to peripheral insulin resistance compensatory hypersecretion from islet cell of pancreas leads to disturbance and hence decline in the islet secretory capacity. Tissues that have high glucose demand mainly skeletal muscle, adipose tissue, liver most prominently demonstrate a reduction in insulin sensitivity (Forbes and Cooper., 2013). In contrast to type 1 form which is more common in children, it is mostly found in people over 40 years of age. However, exceptions exist to the finding as in some countries children are major target of the disease (Zimmet et al., 1990; Eriksson et al., 1991; Glaser., 1997; Scott et al., 1997). A combination of genetic and environmental factors are involved in the establishment and progression of the disease. Heterogeneity seen in genes involved make it difficult to find the susceptible gene (Ozougwu et al., 2013). Environmental factors include obesity, decline in physical activity, shift to unhealthy life style, ageing, drinking, smoking and many more. Many times obesity is seen co-morbid with type 2 diabetes. Even mild obesity increases the disease risk 4-5 times. Increase in visceral mass cause resistance to insulin in obese people (Zimmet et al., 1990; Eriksson et al., 1991; WHO., 1994; Ozougwu et al., 2013). The magnitude of disease, associated symptoms and distribution are found to have geographical differences. Caucasian, Pima Indians, Arabs are more prone to the disease. India ranks high with most of the cases seen in elderly (Knowler et al., 1990; Cook et al., 1993; Shah et al., 2009; Moghissi., 2013; Polinski et al., 2015).

Maturity onset diabetes of the young (MODY)

Maturity onset diabetes of the young is a familial form of NIDDM. It is a form of type 2 diabetes that develops in children, adolescents or young adults of age < 25 years (Raffel et al., 1997). It has a strong genetic background with mutation in glucokinase gene and key glucose metabolising gene serving as a major factor for its
etiology (Hattersley et al., 1992; Froguel et al., 1993). Besides, metabolic, clinical and environmental disturbances are also reported. Among all type 2 diabetes cases, 2-5% are known to have MODY. It is inherited as an autosomal dominant trait. It has geographical disturbances. Found rarely in Caucasians but common in blacks and Indians (Lederman., 1995; Raffel et al., 1997; Ozougwu et al., 2013).

**Symptoms**

A handsome similarity is seen in both type 1 and 2 diabetes. However, a difference in intensity of symptoms varies. More rapid and typical symptoms are seen in type 1 diabetes. However, the onset is insidious in type 2 (Kumar and Clark., 2002). Both types are hyperglycemic and the characteristic features include:

- Polyuria
- Polydipsia
- Polyphagia
- Fatigue
- Weight loss
- Stunted growth
- Susceptibility to opportunistic infection
- Cramps
- Constipation
- Candidiasis
- Tingling sensation or numbness in feet

Prolonged disease course is associated with microvascular or macrovascular complications leading to organ failure. Most commonly targeted organs include eyes, nerve, heart, blood vessels, coronary artery. Comorbidities associated with most cases of type 2 diabetes are atherosclerosis, hypertension, end stage renal disease which ultimately could lead to death (Ishimura et al., 2001; Anonymous., 2004; Michael and Fowler., 2008; ADA., 2009).
Introduction

Diabetic complications

Long standing diabetes is associated with a number of adjoining problems. Onset and progression of diabetic complications is mainly due to hyperglycemia and insulin resistance in both type 1 and type 2 diabetes (Anon., 1993; Jakus and Reitbrock., 2004). Hyperglycemia also leads to AGEs production and they are reported to be elevated in the serum of diabetic people. These complications cause severe problems like coma, ketoacidosis; the most devastating being vascular diseases. They can be categorised into 2 major types namely microvascular and macrovascular complications. Microvascular here refers to small blood vessels. It is a collection of heterogeneous diseases and the major symptoms include retinopathy, neuropathy and nephropathy. As a consequence, blindness, end stage renal disease and debilitating neuropathies may occur. Nephropathy is exhibited as reduced glomerular filtration and if prolonged may result in macrovascular complications. These complications target arteries and cause life threatening cardiovascular damage and cerebrovascular disease manifested as myocardial infarction and stroke (Songer and Zimmet., 1995; Cade., 2008; Forbes and Cooper., 2013). It accounts for 50% of all the diabetes associated death (Leung and Lam., 2000). Besides this, long term diabetes is also associated with depression, dementia and sexual dysfunction (Cukierman et al., 2005; Adeniyi et al., 2011; Nouwen et al., 2011; Thorve et al., 2011). The associated morbidity and mortality also has costly treatment thus posing a burden to authorities concerned with healthcare both in developing as well as developed nations (Jakus and Reitbrock., 2004).

Diabetes and glycoxidation

Diabetes is intimately linked to glycoxidation with glucose being the key player in the disease. An abundance of glucose due to disturbance in insulin level and by other alternative pathways like polyol pathway, hexosamine pathway, protein kinase C (PKC) pathway and others in turn cause hyperglycemia (Brownlee., 2001; Bonnefont-Rousselot., 2002; Spitaler and Graier., 2002). Glucose in turn start interacting with biological macromolecules mainly proteins and after a series of steps form AGEs. Glucose is equally involved in creating oxidative stress. It undergoes auto-oxidation forming reactive dicarbonyl compounds such as 3-deoxyglucosone, glyoxal and methylglyoxal followed by the formation of $\text{O}_2^-$ and $\text{O}_2^-$ rapidly forms...
Fenton’s reaction mediated toxic OH’. Thus, a synergistic interaction is seen between glycation and oxidation, collectively called glycoxidation (Bunn and Higgins., 1981; Wolff and Dean; 1987; Baynes., 1991; Hunt et al., 1993; Lyons and Jenkins., 1997). CML, pentosidine, pyralline and crossline are some fluorescent and non-fluorescent AGEs found in the sera of diabetic patients (Ansari and Dash., 2013a). Studies have reported a 20-30 % and 40-100 % rise in the serum AGEs level in uncomplicated diabetes and diabetes complicated with coronary artery disease and microalbuminuria respectively (Berg et al., 1998; Kilhovd et al., 1999; Tan et al., 2004; Liu et al., 2005). AGEs can have the following fate depending upon the receptor they interact with. AGE-R1, AGE-R3 and scavenger receptors promote renal clearance, however, interaction with RAGE can initiate a plethora of events like intracellular signalling, gene expression which promote release of proinflammatory cytokines and free radicals (Ahmed., 2005; Forbes and Cooper., 2013). Accumulation of AGEs in tissue and organs results in chronic complications like retinopathy, neuropathy, nephropathy and other macrovasculopathies. Immunohistochemical analysis revealed AGE modified protein in atheroma’s (Horie et al., 1997; Schleicher et al., 1999). Nephropathic patients with decreased glomerular filtration rate (GFR) or impaired renal clearance poses difficulty in AGE removal. Thus, high endogenous production and reduced clearance together accounts for AGE toxicity in diabetic patients (Miyata et al., 1999) (Fig. 19).

**Rheumatoid Arthritis (RA)**

**History**

Hippocrates in fourth century B.C first recognised rheumatic diseases. The credit of reporting first series of patients with RA in 1800 goes to Augustin Jacob Landre–Beauvais, a French medical student. However, the term was introduced in 1876 by Sir Alfred Garrod. ‘Rheuma’ in RA means continuous flow of pain through different joints of the body. The appearance and lesions as reported by Goemalre in ancient skeleton gives a clear indication of the disease existence in North America at least 3000 years ago (Goemacre et al., 1990; Storey et al., 1994; Sangha., 2000; Pappas et al., 2008).
Introduction

Fig. 19: AGEs production and clearance from the body
Source Forbes and Cooper., 2013

Definition

This inflammatory autoimmune disorder of unknown etiology is chronic and multisystemic in nature affecting approximately 1% of world’s population. The main characteristic feature being chronic, erosive and symmetrical synovitis of joints and a wide array of multisystem comorbidities (Sangha., 2000; Ling-dong et al., 2008).

Signs and symptoms

This is a disease of joints and mainly targets joints of hand, wrist, knees and feet. The disease begins with inflammation of synovial joint followed by cartilage destruction and bone erosion. The patients experience pain, redness, swelling and difficulty in joint movement (David and Weinblatt., 2001; Sweeney and Firestein., 2004). The symptoms and duration of disease is found variable ranging from mild oligo articular illness for short time with negligible damage of joints to relentless progressive polyarthritis with functional impairment and disability. Besides, subcutaneous nodules, pericarditis, pulmonary nodules, mononeuritis multiplex,
episcleritis are amongst the non-articular features (Sangha., 2000) (Fig. 20). A disturbance in sleeping pattern, fatigue, anxiety decreases the quality of life. Depression is highly co-morbid with RA and 13–42 % of RA patients have a likely occurrence of depression (Bruce., 2008). Recent reports suggest a decline or fail in working ability post 5 year disease onset in approximately 33 % of sufferers, the disability reaches to 50 % post 10 years (Young et al., 2007).

**Fig. 20:** Pathobiology of a normal joint and rheumatoid joint

**Source** Smolen et al., 2016
Diagnostic criteria of RA

The diagnosis is not limited to single disease entity. Despite intensive research, a specific causative agent is not known and so diagnosis is based on combination of laboratory, clinical and radiological abnormalities (Weyand and Goronzy., 2000; Wordsworth., 2001). To monitor the prevalence and progression of RA, American College of Rheumatology had set defined criterion in 1958 (Ropes et al., 1958). It was later revised on the basis of computerised analysis 262 test and control subjects (having other form of RA). The modified criterion was published in 1988 and is called “1987 Revised American Rheumatism Association criteria for classification of Rheumatoid Arthritis” (Arnett et al., 1988).

1. Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement.
2. Arthritis of 3 or more joint areas: At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left proximal interphalangeal (PIP) joints, metacarpophalangeal (MCP) joints, wrist, elbow, knee, ankle, and metatarsophalangeal joint (MTP) joints.
3. Arthritis of hand joints: At least 1 area swollen (as defined above) in a wrist, MCP or PIP joint.
4. Symmetric arthritis: Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPS, MCPs or MTPs is acceptable without absolute symmetry).
5. Rheumatoid nodules: Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician.
6. Serum rheumatoid factor: Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in 4 % of normal control subjects.
7. Radiographic changes: Radiographic changes typical of RA on postero-anterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or mostly marked adjacent to the involved joints (osteoarthritis changes alone do not qualify).
For RA to occur, at least 5 symptoms should last for > 6 weeks. The above mentioned criteria has a sensitivity of 91-94% and specificity of 89% compared to control subjects having any other form of arthritis except RA (Arnett et al., 1988). This criterion lacks sensitivity in initial stages of disease. This shortcoming in the former criterion leads to emergence of a new criterion for RA classification. It was put forward by the joint efforts of working group from ACR and European League Against Rheumatism (ELAR). It focused on defining the disease by its earlier stage features which include:

1. Presence of synovitis in at least one joint.
2. Scores from for domains should sum up to 6 or greater i.e. the score for site and number of joints, serological abnormality, elevated acute phase response and symptom duration should range between 0–5, 0–3, 0–1 and 0–1 respectively (Aletaha et al., 2010).

**Factors affecting RA**

**Environmental factors**

Many non-genetic factors are associated with RA directly or indirectly. Age, sex, income, literacy and many other factors have a profound effect on RA severity. Prevalence is higher in females, lower income groups, unemployed, illiterate and aged people.

**Smoking**

Besides the other harmful effects of smoking, it also has a role in the pathogenesis of RA. Smokers are more prone to the disease as they have increased serum RF. Abnormal function of immune system also leads to infection in such patients (Saag et al., 1997). Some studies also report that RA patients with a smoking history have a greater disability and disease activity than non-smokers. Extra articular manifestations and rheumatoid nodules are also more in such patients. Moreover smokers are also found resistant to anti-rheumatic drugs (Wolfe 2000; Papadopoulos et al., 2005; Manfredsdottir et al., 2006; Mikuls et al., 2008; Westhoff et al., 2008; Soderlin et al., 2011).
Introduction

Diet

Like in other chronic diseases, diet plays an important role in the severity of RA (Pattison et al., 2004a). Fruits, vegetables, fish, olive oil and vitamin C are beneficial in reducing the symptoms of established RA (Shapiro et al., 1996; Trichopolou and Vasilopoulou., 2000). Several studies are conducted to establish association of diet with RA incidence. A case control study in Greek hospital reported direct association between cooked vegetables and reduced role of RA (Linos et al., 1999). Cruciferous vegetables and β cryptoxanthin are beneficial in RA as observed by Cerhan et al (Cerhan et al., 2003). A study conducted in Finland on the effect of coffee on RA found that risk of developing RA increased with more than 4 coffee/day. Opposite results were found for tea. Besides these, alcohol and meat are also causative factors for RA (Pattison et al., 2004b).

Vitamin D

It has a protective role in RA. Less vitamin D consumption is related to disability associated with RA. A study conducted on murine model showed supplementation of vitamin D declined disease severity. It acts as an immunosuppressant, reducing T cell responsiveness during active immune response (Merlino et al., 2004; Patel et al., 2007; Haque et al., 2010; Kerr et al., 2011).

Tobacco

Consumption of tobacco increases the risk of RA, the association of two was first published in 1987 and since then many more studies were conducted and found positive correlation between tobacco and RA (Pladevall-Vila et al., 1996; Symmons et al., 1997; Hutchinson et al., 2001; Krishnan et al., 2003; Padyukov et al., 2004). It acts as an immunosuppressant affecting both humoral and cell mediated immune response. Its immunosuppressive action can be best demonstrated by its inhibitory effect on the release of cytokines mainly IL-1β, IL-2, IL-8, gamma interferon mainly by endothelial cells. Besides macrophage mediated destruction of intracellular cytokines also seems to be declined (Sopori and Kozak., 1998; Arnson et al., 2010).
**Infectious agents**

A variety of infectious agents are involved in the initiation of chronic inflammatory diseases like RA. Evidences indicate mycoplasma and viral agents as causative factors of the disease (Bennett., 1978). Infectious agents mediated arthritis can be categorised into following ways:

1. Multiplication of infectious agent within the joint cavity. Certain viruses (small pox), bacteria (pyrogenic bacteria, mycobacteria) and fungi come in this category (Bennett., 1978).
2. Localisation and hence initiation of immune response in joint space examples include herpes simplex in rabbit and guinea pig and mycoplasma hyorhinis in swine (Ogra and Herd., 1971).
3. Distantly located infectious agent is able to cause arthritis eg: Reiter’s syndrome (Catterall., 1976).
4. Arthritogenic toxins released by infectious agents as a causative factor for arthritis eg Arbovirus infection (Smith and Sanford., 1967).

**Gender**

Women are more likely to develop the disease as surveyed by Norwich Health Authority. However, post menopause, the chances of disease onset is similar in both sexes. The major factor of this gender difference is hormonal and reproductive features. Woman on oral contraceptives have lower incidence of RA, an observation by Royal College of General Practitioners in 1978 (Wingrave and Kay., 1978). Influence of hormone is also found in males as reduced testosterone levels are found in RA afflicted man (Cutolo et al., 1984; Gordon et al., 1986; Spector et al., 1988; Spector et al., 1989). Even decreased testosterone and dehydroepiandresterone (DHEA)-sulfate are found in woman with RA (Feher et al., 1986; Sambrook et al., 1988). However, some studies provide conflicting results (Dougodos et al., 1983; Cutolo et al., 1986; Spector et al., 1987).

**Genetic factors**

Prevalence of RA has a strong genetic association, linked to MHC region, a region 3.6 MB located on chromosome 6 and having around 220 genes (Newton et al.,
Introduction

It has 3 sub regions called class I, II and III. It is the HLA-DRB1 region on MHC that is in particular linked to RA. The link is mapped to a unique amino acid sequence “QKRAA” from amino acid 70 to 74 called shared epitope present on third hypervariable region of DRB1 chain (Hughes et al., 2008). Alleles carrying this sequence are DRB1*0401, *0404, *0405, *0408, *0101, *0102, *1402, *09 and *1001, are all positively associated with RA (Gregersen et al., 1987; Weyand et al., 1992; MacGregor et al., 1995). Besides some non MHC genes such as genes coding for corticotropin releasing hormone (CRH), oestrogen synthesis, IFN-γ, and other cytokines are also associated with RA (Hajeer et al., 1998., John et al., 1998; John et al., 1999; Fife et al., 2000; Khani-Hanjani et al., 2000; Ollier 2000; John et al., 2001; Pokorny et al., 2001).

Epidemiology of RA

Regional variations exist in the prevalence of RA accounting for 0.5–1 % of cases throughout the world. The incidence varies in different populations with a high prevalence in North America and North Europe compared to other regions. A time dependent decline post 1960 is also reported in North America, North Europe and Japan (Alamanos and Drasos., 2005). Another study conducted, reported a high incidence in Pima Indians and Chippewa Indians in comparison to China and Japan (Silman and Pearson., 2002).

Autoantibodies in RA

Rheumatic autoimmune disease is found to have a variety of autoantibodies in the serum and synovial fluid. Amongst them, some autoantibodies are proven extremely useful and seems to play a major pathogenic role thus proven to be extremely useful diagnostic tools for disease (Von Muhlen and Tan., 1995). The target autoantigens represent cartilage components, stress proteins, citrullinated proteins, enzymes etc. The spectrum of autoantigens varies in different patients and so the autoantibodies (Blass et al., 1999). Till now, following autoantibodies are reported in RA patients.

- Rheumatoid factor (RF)
- Anti citrullinated peptide antibodies (ACPA)
- Anti - A2/ Anti RA33 antibodies
- Anti Sa
- Anti Immunoglobulin binding protein (BiP)
Rheumatoid factor (RF)

RF represents most studied immunological hallmark of RA. This established serological marker is an integral part of definition of this disorder and is used for RA detection since last 60 years. RF autoantibodies, usually of IgM isotype are targeted to Fc portion of IgG. It has a favourable sensitivity profile of 60–80 % (Tighe and Carson., 2001; Steiner and Smolen., 2002), however, due to a low specificity (approximately 66 %), search for other autoantibodies and their target autoantigen useful for RA diagnosis begin. As per observation, RF occur less frequently and are of low titre in early disease stages. Besides RA, RF are also reported in other diseases like sjogrens syndrome, mixed connective tissue disease and systemic infections (SLE) (Tighe and Carson., 2001; Nell et al., 2005). Osteoarthritis, chronic infections, old age have low titre RF (Smolen., 1996; Tighe and Carson., 2001). RF is also found in 10 % of normal individuals but they differ considerably than those found in RA patients. The major differences are stated below:

In normal individuals, they are released from CD5+ B cells, have low affinity for IgG as they have mechanism to prevent affinity maturation, are polyreactive, have low ratio of replacement to silent mutation in their CDRs. In contrast, RF in RA synovium are of high affinity and their CDRs have multiple replacement mutation pointing to their dependence on T cells. T cell help in RF synthesis can also be demonstrated by a study conducted on transgenic mouse model, in which RF synthesis is driven by CD40 signalling (Mantovani et al., 1993; Borretoen et al., 1997; Kyburz et al., 1999).

As per the observations it can be concluded that in normal subjects, a strict control is maintained on the emergence of high affinity RF. The main function of these autoantibodies is immune complex formation and complement fixation (Song and Kang., 2010).

Anti citrullinated peptide antibodies (ACPA)

ACPA represents a group of new autoantibodies with 70–90 % sensitivity and 90 – 95 % specificity. Owing to their high sensitivity and specificity, their diagnostic value is much more than RF. Unlike RF, their likely occurrence in other disease states and healthy individuals is negligible. They are associated with erosive RA (Kroot et al., 2000; Van der-Helm-van et al., 2005; Im et al., 2009). They are referred as antikeratin, antifilaggrin, anti-perinuclear factor (APF) depending upon the target antigen. ACPA’s are directed to a multitude of antigens such as deiminated form of α and β
fibrin, citrullinated fibrinogen, citrullinated enolase, citrullinated eukaryotic translation initiation factor 4G1 as well as citrullinated peptides of collagen I and II (Masson-Bessiere et al., 2001; Kinloch et al., 2005; Koivula et al., 2005; Okazaki et al., 2006; Takizawa et al., 2006). Citrulline is the common critical constituent of the antigenic determinant of all these antibodies. It is formed by Ca\(^{2+}\) dependent peptidyl arginine deiminase (PADI) mediated deimination of arginine. Antikeratin were first discovered in 1979. The target antigen is “intermediate filament aggregating protein fillagrin” expressed in keratinised epithelial cells. APF was first described more than 30 years ago by demonstration of a perinuclear staining pattern with RA sera using indirect fluorescence on buccal mucosal cells. They recognise profilaggrin and fillaggrin antibodies respectively (Simon et al., 1993; Schellekens et al., 1998; Girbal-Nenhauser et al., 1999; Gussin et al., 2001). Like RF, ACPA also forms immune complexes. A study conducted on collagen induced arthritis model contradicts ACPA dependence on citrullination as the patients do not have ACPA despite citrullination in inflamed joints. So, it was later said that ACPA production is limited to subjects with a genetic background. The predominant genetic factor being the shared epitope (SE), located in HLA-DRB1 gene (Vossenaar et al., 2003; Berglin et al., 2004; Van Gaalen et al., 2004; Kaltenhauser et al., 2007). Besides some non SE-HLA alleles like 185 8c/T allele polymorphism of PTPN22, several single nucleotide polymorphism of PADI4, 158V/F polymorphism of Fc \(\gamma\) receptor III A gene and others also induce ACPA production (Iwamoto et al., 2006; Feitsma et al., 2007; Kokkonen et al., 2007; Vignal et al., 2009).

**Anti-A2/ anti RA 33 Antibodies**

These antibodies are directed to 33 KDa heterogeneous ribonucleoprotein (hn-RNP) involved in transport and splicing of m-RNA. Different tissues have different expression level, highest being in synovial tissue. These antibodies are detected in early disease stages, when the sera are RA negative. 33 % of RA patients are found positive for these antibodies. Besides RA, they are also found in SLE (20–30 %) and MCTD patients (40 %). They have a low sensitivity but high specificity as other arthritides like osteoarthritis, psoriatic arthropathy, reactive arthritis are anti A2/RA33 negative (Richter et al., 1998; Dumortier et al., 2000).

**Anti Sa**

These antibodies are directed to a 50 KDa citrullinated antigen isolated from human tissues like spleen, placenta, RA synovium. 40 % of RA patients are positive for the antibody. They have 92-98 % specificity profile (Despres et al., 1994; Hueber et al., 1999).
Anti-Immunoglobulin binding protein (BiP)

The target antigen is chaperone of endoplasmic reticulum (ER) localised immunoglobulin heavy chain binding protein (BiP), a stress protein. BiP is a glucose regulated protein of heat shock protein family and maintains homeostasis in normal as well as stress conditions (Blass et al., 2001; Pockley., 2001). Due to their high specificity (96%), these antibodies are useful in the diagnosis of RA. However, sensitivity profile is only 60%. As a result of high expression in RA synovium, they become a target of autoreactive T cells leading to autoimmune response (Blass et al., 1995; Blass et al., 2001).

Pathogenesis of RA

RA is a complex phenomenon in which abnormal immune system release destructive and abnormal molecules that mainly target the joint space, the point of contact between two articulating bones surrounded by a capsule. It as a 3 layered protective covering which encloses a cavity called synovium (Ainola et al., 2005; Mobasher., 2011). It is the synovium which is majorly involved in RA pathogenesis. The accumulated immune cells mediate a multitude of destructive processes resulting in fibrosis, synovial cell proliferation, cartilage and bone erosion, pannus formation etc. Pannus is synovial thickening resulting from inflammation mediated enlargement of synovial cells. It has a multitude of destructive effects mainly on cartilage and soft subchondral bone. Besides, it aids in the release of cytokines, proteolytic enzymes and prostanoids. IL-1 is the predominant cytokine and accounts for the perpetuation of inflammation by release of neutrophils, macrophages at the site. It is also involved in the proliferation of lymphocytes (Dinarello., 1996; Bresnihan., 1999; Mobasher., 2011). Cross reactivity of lymphocytes with numerous synovial products contributes to chronic inflammation, cytokine and inflammatory mediators release. Mainly 4 such mediators TNF α, Interleukins, TGF-β, PDGF dominates the cavity. T and B lymphocytes release antibodies and it is the B cells that release Rheumatoid factor, the immunological hallmark of RA. It is involved in immune complex formation and complement fixation. IL-1 also triggers the release of IL-6 and matrix metalloproteinases (MMPs). MMPs further contributes in cartilage destruction. Also, the inflammatory response leads to oxidative stress releasing ROS and heat shock proteins which further aggravates the process. The vicious cycle continues ultimately leading to destruction of cartilage, tendon, ligament, bones and blood vessels (Carol and Hani., 2004; Benucci et al., 2005).
**Introduction**

**Objectives of the study**

Glycoxidation is a collective term used for two interrelated post translational modifications i.e. glycation and oxidation. MG is a highly potent glycating agent with 20,000 times more reactivity than glucose. As ROS are known to be produced during both formation and degradation of MG; understanding the implication of glycation and oxidation on the structural integrity of protein as well as its role in disease pathogenesis becomes important. The present study has aimed to investigate the role of glycoxidatively modified IgG (i.e. OH’ treated MG glycated IgG) in diabetes and RA using IgG. The objectives of our studies were

1. To study physicochemical and immunological changes in IgG following glycoxidation (OH’ modification of glycated IgG).
2. To evaluate the presence of antibodies against glycoxidatively modified IgG in the sera of type 2 diabetes and RA patients.

The objectives were achieved using the following strategy:

IgG isolated from healthy human serum was first glycated with MG (24 hrs, pH-7.4) followed by its modification with OH’ (a highly potent ROS generated in our laboratory by Fenton’s reaction) (30 min, pH-7.4). The structural changes were monitored by UV absorption spectroscopy, fluorescence spectroscopy, CD spectroscopy, FTIR spectroscopy, SDS-PAGE, ANS fluorescence, DLS, DSC, LCMS and MALDI-TOF. The free sulphydryl group and carbonyl content were also quantitated in native and modified forms of IgG. ThT binding assay, CR absorption study and staining assay, SEM and TEM were carried out to study aggregation kinetics in native and modified IgG. The antigenicity of native and glycoxidatively modified IgG (i.e. OH’ treated MG glycated IgG) was probed in female rabbits. The specificity of induced antibodies was evaluated by competitive inhibition ELISA and gel retardation assay. Furthermore, possible role of glycoxidatively modified IgG (OH’-MG-IgG) in the pathogenesis of diabetes and RA was investigated by analyzing the presence of auto-antibodies against OH’-MG-IgG in the sera of these patients. To further confirm the presence of glycoxidatively modified IgG in diabetes and RA patients; structural changes in the IgG from these patients were monitored physicochemically. Experimentally induced anti-OH’-MG-IgG antibodies have been used as immunochemical probe to confirm the glycoxidatively modified epitopes on the IgG obtained from diabetes and RA serum samples.