

Review

Chemistry of Hydrogen Peroxide Formation and Elimination in Mammalian Cells, and Its Role in Various Pathologies

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Abstract: Hydrogen peroxide (H₂O₂) is a compound involved in some mammalian reactions and processes. It modulates and signals the redox metabolism of cells by acting as a messenger together with hydrogen sulfide (H₂S) and the nitric oxide radical (•NO), activating specific oxidations that determine the metabolic response. The reaction triggered determines cell survival or apoptosis, depending on which downstream metabolic pathways are activated. There are several ways to produce H₂O₂ in cells, and cellular systems tightly control its concentration. At the cellular level, the accumulation of hydrogen peroxide can trigger inflammation and even apoptosis, and when its concentration in the blood reaches toxic levels, it can lead to bioenergetic failure. This review summarizes existing research from a chemical perspective on the role of H₂O₂ in various enzymatic pathways and how this biochemistry leads to physiological or pathological responses.

Keywords: hydrogen peroxide H₂O₂; metabolic response; induced pathologies



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1. Introduction

Hydrogen peroxide H₂O₂, also known as dioxygenane or dioxygen, is a strongly oxidizing compound with a particularly unpleasant odour that decomposes into oxygen and water, releasing large amounts of heat. Although non-flammable, it is a strong oxidizer that can cause combustion when it comes into contact with organic material or metals such as copper, silver or bronze [1].

Because of its chemical properties, hydrogen peroxide is used in many areas of human activity, such as healthcare, as antiseptic, antimicrobial and antibacterial agent [2].

In mammals, in terms of redox signalling and regulation, H₂O₂ is an endogenous oxidant [3]. It is the most stable of the reactive oxygen species ROS, clearly involved in the regulation of protein function [4], and under certain circumstances is also a precursor of hydroxyl radical •OH and hypochlorous acid HOCl [5].

At micromolar concentrations, H₂O₂ is reactive, and at high concentrations it can damage energy-transforming cell systems, e.g., by inactivating the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase [6]. In the Fenton reaction, soluble Fe(II) donates an electron to an H₂O₂ molecule, causing it to decompose into hydroxyl radicals •OH + [−]OH, which react at diffusion rates and can randomly oxidise virtually any organic molecule. Hydroxyl radicals kill by DNA damage [7].

Until a few decades ago, H₂O₂ was considered an undesirable and harmful product for metabolism because it is a by-product of oxidative stress in cells [8]. Instead, it has

come to the fore in recent years as a key redox signalling molecule in a variety of biological processes, including cell differentiation and proliferation, inflammation, tissue repair, circadian rhythm, and even ageing [9]. Because of its relative stability, with a cellular half-life of around 10^{-3} s, its diffusivity and its selective reactivity, H_2O_2 is often presented as the most important redox signal molecule [10].

H_2O_2 can act as a signalling molecule or, conversely, cause oxidative damage to biomolecules (a condition known as oxidative stress). This ambivalence depends on the cellular context, the local concentration of H_2O_2 and the kinetics of its production and elimination [11]. Under healthy steady-state conditions, their production and elimination are balanced, but under conditions of oxidative stress, superoxide anion $\bullet O_2^-$ and thus H_2O_2 , are overproduced.

Hydrogen peroxide is formed as a product of the monovalent reduction of superoxide or by the divalent reduction of oxygen [12]. The main source of hydrogen peroxide is enzyme-catalysed superoxide dismutation, but it can also result from the two-electron reduction of oxygen in reactions catalysed by oxidases such as xanthine oxidase, glucose oxidase, amino acid oxidase, urate oxidase, and others. Since hydrogen peroxide is uncharged with a $pK_a \approx 10.8$ at neutral pH, it can easily penetrate biological membranes [13]. Hydrogen peroxide is a strong oxidant, but due to its slow reaction kinetics with the biomolecules, it is relatively unreactive. Therefore, it can accumulate in cells and tissues at relatively high concentrations [14]. H_2O_2 is capable of directly oxidize many molecules and inactivating certain enzymes with thiol groups or methionine residues in their active site [15]. Hydrogen peroxide is a weak reducing agent, with an $E^\circ' (\bullet O_2^-, 2H^+ / H_2O_2)$ of +0.940 V, and an oxidizing agent with an $E^\circ' (H_2O_2, H^+ / H_2O, HO^-)$ of +0.320 V.

In the following sections, we review the mechanisms of production and elimination of H_2O_2 , particularly in episodes of inflammation, cancer and several diseases. We also examine recent trends and techniques for the detection of H_2O_2 in blood, urine or exhaled air.

2. Hydrogen Peroxide Formation

It has been known for more than 60 years that hydrogen peroxide forms naturally in living organisms. H_2O_2 is not a free radical, but it is a reactive form of great importance because it can form the hydroxyl radical $\bullet OH$ in the presence of metals such as iron, in the known Fenton reaction. In mammalian cells, two forms of H_2O_2 production coexist, defined as enzymatic and non-enzymatic generation. In essence, the non-enzymatic one derives from the reduction by e^- and H^+ of the $\bullet O_2^-$ anion, which in turn comes from the reduction of O_2 in the mitochondrial respiration pathway (complexes I to IV) and which takes place in the cellular mitochondrial matrix.

The enzymatic pathway starts from the $\bullet O_2^-$ anion, and involves the enzyme superoxide dismutase, SOD1, SOD2 and SOD3. These mechanisms are explained in detail in the following subsections.

2.1. Non-Enzymatic Generation of H_2O_2

ROS are also formed in the mitochondrial matrix by partial reduction of O_2 to $\bullet O_2^-$ and subsequent reduction of the superoxide radical by e^- and H^+ to H_2O_2 and H_2O . Until a few years ago, this "leakage" of electrons from the chain was considered an altered process, however, it has now been proposed that mitochondrial $\bullet O_2^-$ and H_2O_2 may be involved in redox reaction-dependent signalling processes [16], as well as in the biological clock of ageing [17], and even act as an indicator of the proper functioning of the electron transport chain ETC. Inside mitochondrial matrix, the harmful effects of ROS include structural changes on mitDNA and oxidation of lipids and proteins that perform numerous functions. It is therefore important to keep ROS levels at physiological values, preventing them from increasing and triggering oxidation of biological molecules.

In this route, the final product is H_2O , so O_2 , $\bullet O_2^-$ anion and hydrogen peroxide, in the presence of e^- and H^+ , will also be spontaneously reduced to the final H_2O stage. The Gibbs free energy of this reduction process is, at each step, negative.

Electrons derived from mitochondrial metabolism flow through complexes I-IV of the ETC in the mitochondrial inner membrane. The energy gradient of this process is used to pump protons (H⁺) to the intermembrane space. Mitochondrial respiration is coupled to ATP synthesis via ADP phosphorylation. Protons introduced by ATP synthase are used to reduce molecular O₂ to $\cdot\text{O}_2^-$, H₂O₂ and finally H₂O, Figure 1:

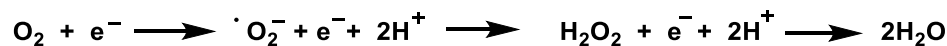


Figure 1. O₂ reduction to $\cdot\text{O}_2^-$ anion, H₂O₂ and finally H₂O.

2.2. Enzymatic Generation of H₂O₂

$\cdot\text{O}_2^-$ is also formed in other cell organelles such as the endoplasmic reticulum, peroxisomes, and cytosol [18] and H₂O₂ production involves the enzyme superoxide dismutase, SOD-1, -2 and -3, Figure 2. In fact, there are enzymatic reactions within the cell that result in the generation of $\cdot\text{O}_2^-$.

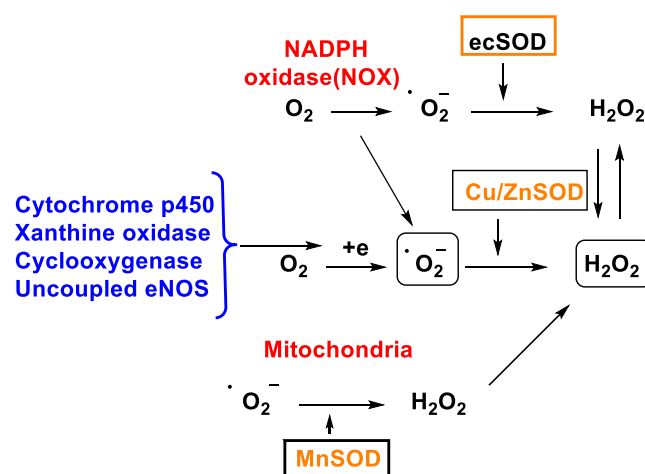


Figure 2. H₂O₂ production through different mechanisms.

In 1968, McCords and Fridovich discovered the enzyme superoxide dismutase SOD isolated from erythrocytes. SOD is the only enzyme capable of detoxifying the superoxide $\cdot\text{O}_2^-$ and is present at the mitochondrial level as well as in the cytoplasm and extracellular space [19]. There are three isoforms, SOD1 (Cu/Zn SOD) is the predominant $\cdot\text{O}_2^-$ scavenger and is localized in the cytoplasm, mitochondrial intermembrane space, nucleus, and lysosomes; SOD2 (Mn SOD) and SOD3 (Cu/Zn SOD) are localized in the mitochondrion and extracellular matrix respectively [20]. This group of metalloenzymes catalyses the dismutation of the superoxide radical to hydrogen peroxide and oxygen, Figure 3:

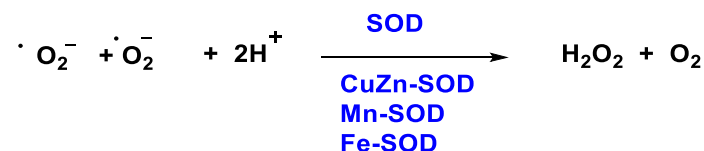


Figure 3. H₂O₂ production through SOD enzyme.

The SOD-catalysed dismutation of the superoxide radical can be represented as the following half-reactions, Figure 4:

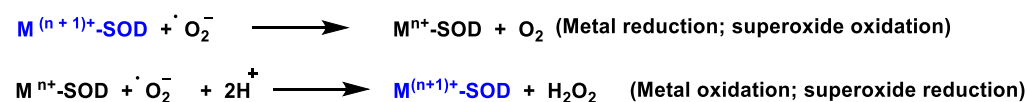


Figure 4. SOD-catalysed dismutation of the $\cdot\text{O}_2^-$.

where $M = \text{Cu}$ ($n = 1$); Mn ($n = 2$); Fe ($n = 2$). In this reaction the oxidation state of the metal cation ranges between n and $n + 1$.

In the catalytic cycle of cytosolic superoxide dismutase Cu/Zn SOD, which contains Cu and Zn in the catalytic site, SOD transforms the $\cdot\text{O}_2^-$ to O_2 by reducing Cu(II) to Cu(I) in its active site. Then, another $\cdot\text{O}_2^-$ molecule causes the oxidation of Cu(I) to Cu(II), generating an H_2O_2 molecule at the end of the cycle. Zinc does not act in the catalytic cycle, it only helps to stabilize the enzyme, Figure 5, left. The catalytic cycle of mitochondrial superoxide dismutase Mn-SOD is similar, except for the use of Mn in the oxidation-reduction reactions, transiting between (Mn(III)) and (Mn(II)) [11], Figure 5, right.

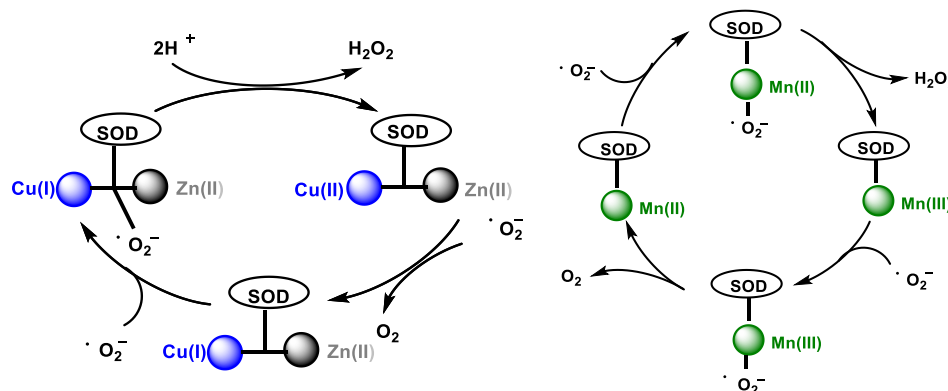


Figure 5. (Left): Cu/Zn SOD catalytic pathway. (Right): Mn SOD catalytic pathway.

NADPH oxidase is an enzyme that catalyses the reduction reaction of O_2 to $\cdot\text{O}_2^-$ and/or H_2O_2 using NADPH as an electron donor [21]. It was first identified in phagocytic cells of the innate immune response and is involved in the “respiratory burst”, generating large amounts of $\cdot\text{O}_2^-$ to destroy invading pathogens.

In general, phase 1 enzymes can transform multiple substrates and catalyse different reactions. They are catalytic proteins of a very diverse nature including enzymes with monooxygenase activity, such as cytochrome P450s or flavin monooxygenase, various oxidases (alcohol dehydrogenase, aldehyde dehydrogenase, amino oxidases, aromatases), epoxide hydrolase, or hepatic and plasma esterases and amidases. Cytochrome P450s are undoubtedly the most prominent member of this group of enzymes and the one that has been most extensively studied [22].

Cytochrome P450s, responsible for detoxifying substances in the body, can carry out oxidation reactions that result in the generation of superoxide [23]. On the other hand, the enzyme xanthine oxidase catalyses the oxidation of hypoxanthine and xanthine to uric acid with the formation of hydrogen peroxide. Thus, the presence of enzymes whose activity leads to the formation of $\cdot\text{O}_2^-$ and H_2O_2 indicates that the production of these species is not a random event and that their production in the cell must therefore have a specific purpose beyond causing damage [14].

In humans, xanthine oxidase is normally found in the liver and not free in the blood [24]. The enzyme xanthine oxidase is a molybdoflavoenzyme, a flavo-protein one (FAD, as a cofactor) containing one molybdenum atom and four ferro-sulphur centres in its prosthetic group [25]. Mo is pentacoordinate. The main function of this metalloenzyme is to hydroxylate a number of substrates, such as hypoxanthine, which is converted to xanthine, which in turn is converted to uric acid. O_2 , which acts as an oxidant in both reactions, is converted to H_2O_2 . At each oxidation step, xanthine oxidase XOR generates superoxide radical ion and hydrogen peroxide [26], Figure 6:

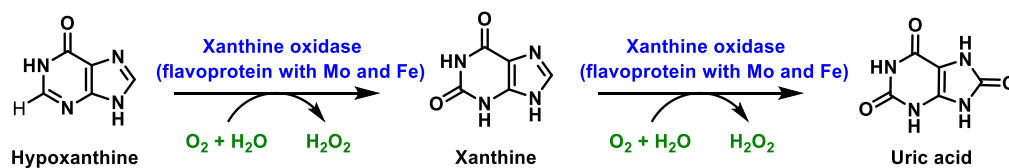


Figure 6. Xanthine oxidase XOR generates superoxide radical ion and hydrogen peroxide.

In mammals, this enzyme catalyses the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid [27]. XOR uses hypoxanthine and oxygen (as an electron acceptor) to give rise to xanthine (eventually uric acid) and superoxide radical. In the active form of XOR, Mo forms two single bonds with the sulphur atom (thiol groups), two bonds with the oxygen atom (one with the oxo group and one with the hydroxyl group) and the fifth coordination position is occupied by a double bond with the sulphur atom. XOR converts xanthine to uric acid, Figure 7, the end product of the catabolism of purine bases in humans.

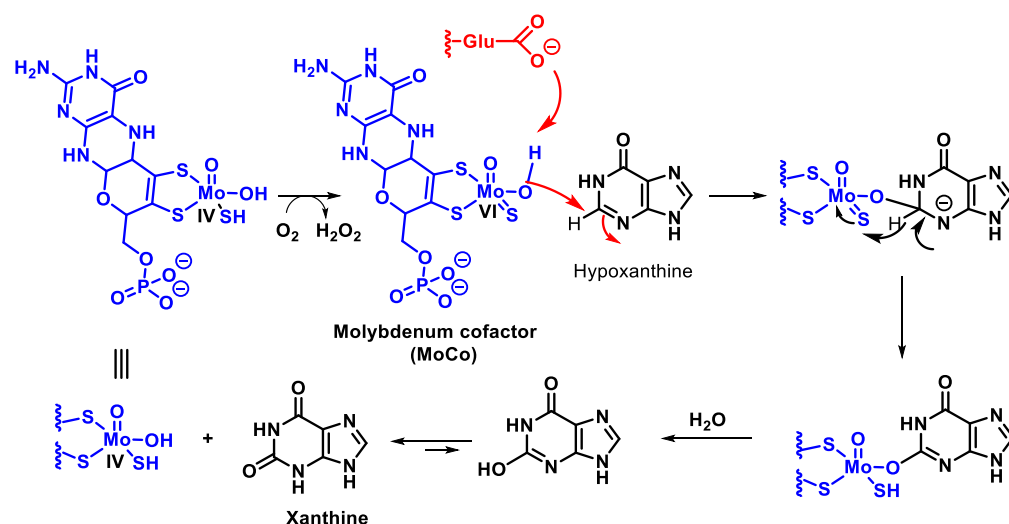


Figure 7. Mechanism of xanthine oxidase reaction.

The mechanism by which XOR converts xanthine to uric acid is not fully understood, but it has been suggested that a reduction and oxidation reaction occurs, as shown in the figure, resulting in oxidative damage to tissues [28], Figure 8.

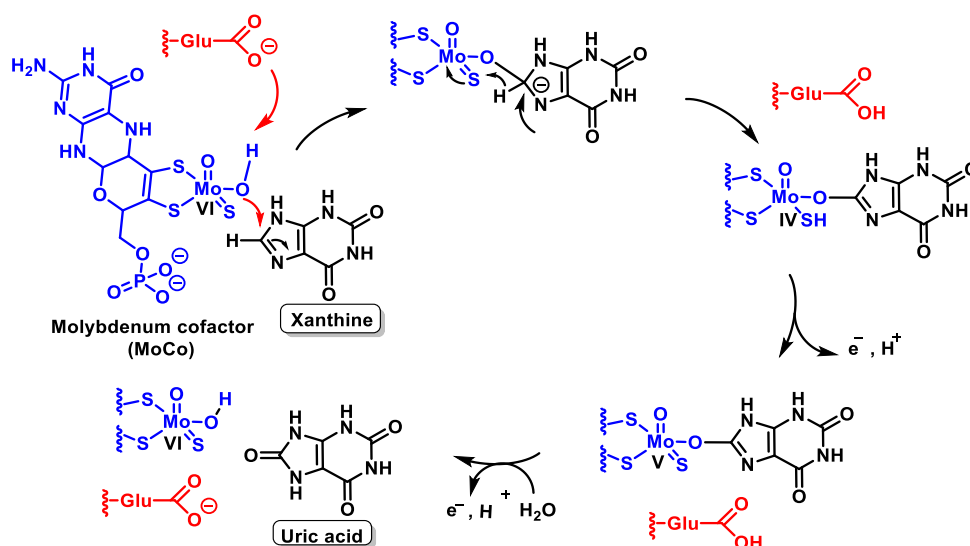


Figure 8. Possible mechanism by which XOR converts xanthine to uric acid.

Catalysis is initiated by base-assisted nucleophilic attack of the equatorial Mo-OH at the C-8 carbon of xanthine with hydrogen transfer from C-8 to MoS and moves from Mo=S to Mo-SH, which simultaneously results in the reduction of Mo(VI) to Mo(IV). Reoxidation of the molybdenum centre occurs by electron transfer to the other redox active sites of the enzyme, accompanied by deprotonation of the Mo-SH bond and cleavage of the bound product by hydroxide from the solvent to regenerate the Mo-OH group.

The peroxisome contains different proteins that can generate H_2O_2 , such as urate oxidase, 1- α -hydroxyacid oxidase, and D-amino acid oxidase.

Peroxisomes are very common cytoplasmic organelles in the form of vesicles with a single membrane found in most eukaryotic cells. They are an oxidative organelle in which molecular oxygen serves as a co-substrate for the formation of hydrogen peroxide. Peroxisomes are named for their ability to produce hydrogen peroxide [29]. In addition, some microorganisms such as bacteria and mycoplasmas release hydrogen peroxide, which can cause damage to the host at the cellular level due to its ability to penetrate biological membranes.

Although urate oxidase is present in almost all living organisms, bacteria, fungi, plants, and animals, it is absent in many primates and in humans. In the human genome, there is a gene for urate oxidase that has been rendered non-functional by two mutations. Since urate oxidase is missing, uric acid is the end product of purine catabolism in humans [30,31].

Unlike other animals, humans do not have the enzyme urate oxidase [31] that converts uric acid to allantoin, a very soluble biomolecule, so they cannot break down excess uric acid, which is very poorly soluble. As a result, urate, which makes up most of the uric acid in the blood, is transported by plasma proteins such as albumin and alpha-globulins [32], Figure 9.

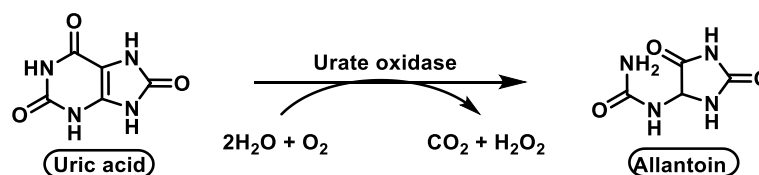


Figure 9. The enzyme urate oxidase converts uric acid into allantoin.

Previously, this metabolic pathway was thought to be carried out by a single enzyme, urate oxidase, but recent research has shown that two other enzymes are involved in this metabolic pathway, Figure 10. The metabolic pathway is initiated by urate oxidase, which produces the unstable 5-hydroxyisourate HIU, followed by hydrolysis by HIU hydrolase to form OHCU, which is also spontaneously degraded.

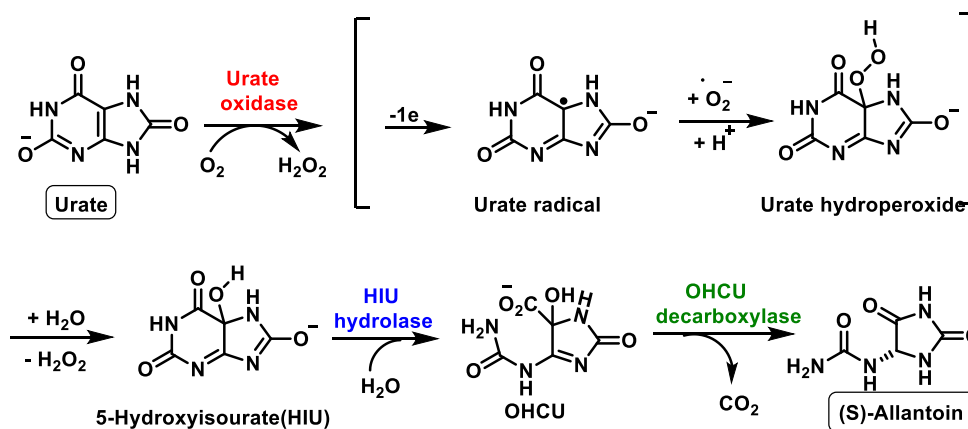


Figure 10. Schematic representation of the ureide pathway.

The third enzyme, OHCU decarboxylase, catalyses the de-carboxylation of OHCU, producing (S)-allantoin in a stereospecific manner [33], Figure 11.

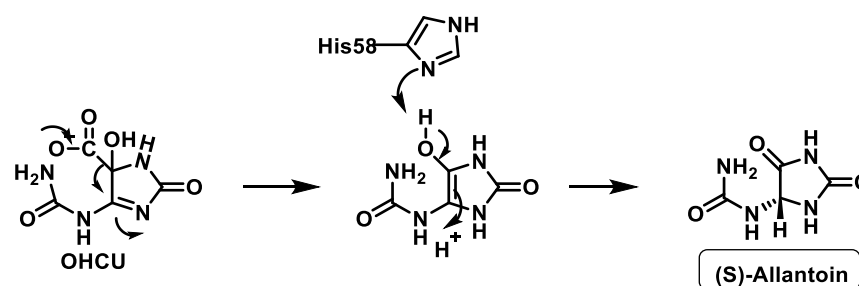


Figure 11. Proposed mechanism of OHCU decarboxylase.

The oxidoreductase enzyme oxidoreductin1 Ero1 catalyses the formation and isomerisation of protein disulphide bonds in the endoplasmic reticulum ER of eukaryotic cells. This luminal glycoprotein is tightly associated with the ER membrane and is essential for the oxidation of protein dithiols. Disulphide bond formation is an oxidative process and Ero1 is required for the introduction of oxidant equivalents into the ER and their direct transfer to the protein disulphide isomerase PDI. Ero1 is a major producer of H_2O_2 in the lumen of the ER endoplasmic reticulum [34]. Oxidative protein folding in the ER is an essential function of eukaryotic cells and requires the transmission of electrons between the different protein components. Ero1 reduces O_2 to H_2O_2 , its activity is allosterically and transcriptionally regulated by the response to unfolded ER proteins. The oxidative activity of Ero1 is linked to H_2O_2 production and consequently burdens cells with potentially toxic reactive oxygen species, so deregulated Ero1 activity impairs cell metabolism under certain conditions of oxidative ER stress [34].

3. Removal of Hydrogen Peroxide

In biological systems, H_2O_2 can be removed directly by the enzyme CAT, Figure 12, which uses H_2O_2 to generate molecular oxygen and water, whereas GPx uses H_2O_2 and reduced glutathione (GSH) to form water and oxidised glutathione GSSG. Fe(II) reacts with H_2O_2 in a Fenton reaction, producing $\bullet\text{OH}$ and OH^- . Iron can also react with H_2O_2 to produce OOH^- and H^+ [35].

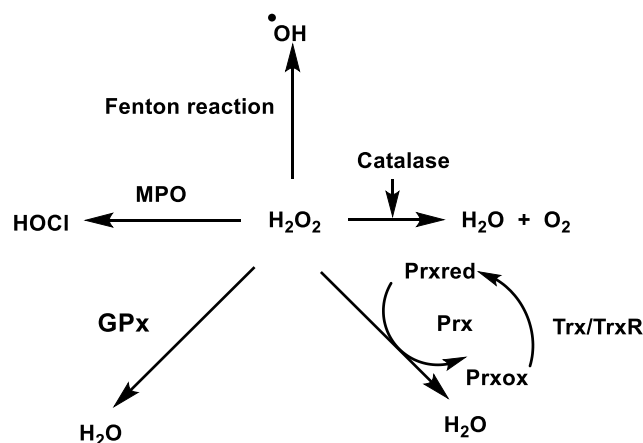


Figure 12. Enzymatic removal of hydrogen peroxide in biological systems.

While H_2O_2 is less reactive and more stable than $\bullet\text{O}_2^-$ [36], Figure 13, it generates hydroxyl radicals, one of the most reactive oxygen species known. Therefore, the removal of peroxide is of utmost importance to avoid oxidative damage.

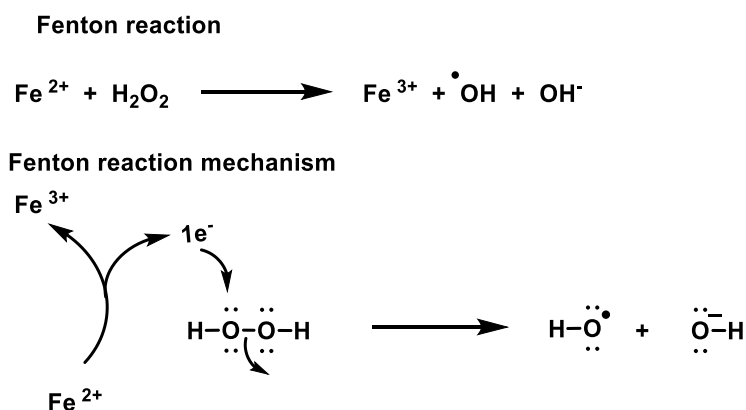


Figure 13. Mechanism of the Fenton reaction.

Non-enzymatic removal of H₂O₂ occurs via the Fenton reaction and enzymatic removal is carried out by catalases, glutathione peroxidases and peroxiredoxins.

3.1. Catalase

Catalase is an antioxidant enzyme found in most aerobic organisms. It catalyses the dismutation of H₂O₂ into water and oxygen. Most of these enzymes are homotetramers with a heme group on each subunit. In the catalase reaction, a two-electron transfer occurs between two hydrogen peroxide molecules, one acting as an electron donor and the other as an electron acceptor. The reaction mechanism proceeds in two steps. In the first step, catalase is oxidised by a peroxide molecule to form an intermediate called compound I. Compound I is characterised by a ferroxyl group containing FeIV and a porphyrin cation radical. In this reaction, a water molecule is formed (reaction 1). In the second step of the reaction, compound I is reduced by another peroxide molecule, returning the catalase to its initial state and producing water and dioxygen (reaction 2), Figure 14.

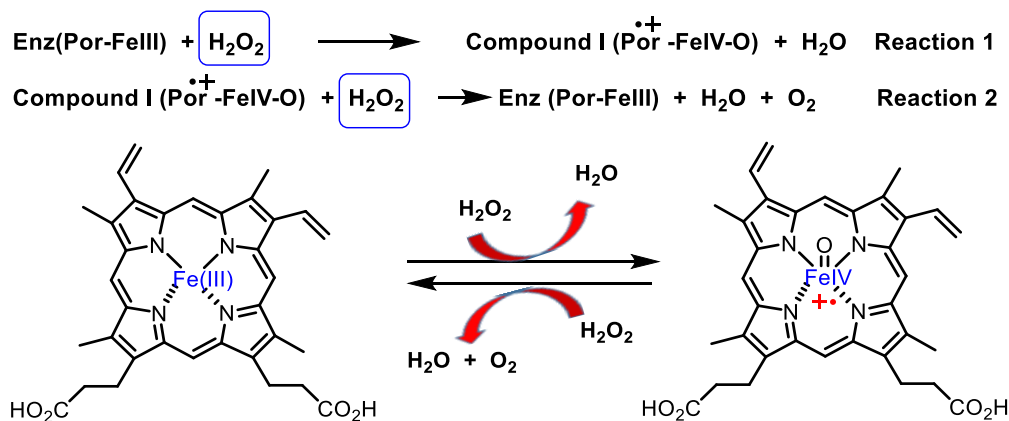


Figure 14. Catalase mechanism of hydrogen peroxide dismutation to water and oxygen.

Catalase is mainly localized to peroxisomes by the Pex5-mediated import pathway [37], as well as in the mitochondrial matrix of some metabolically highly active tissues, such as the heart and liver [38].

3.2. Glutathione Peroxidases GPx

It is in the cytosol and mitochondria. Its importance lies in the fact that it is the main antioxidant system at low levels of oxidative stress [39]. The term glutathione peroxidase GPx is associated with a family of multiple isoenzymes GPx1–8 that catalyse the reduction of H₂O₂ to water using glutathione as an electron donor [40,41]. The different isoforms can be divided into Se-dependent and Se-independent isoforms. The Se-independent form, also

known as glutathione S-transferase GST, catalyses the detoxification of various xenobiotics, with the Se atom not involved in the catalytic reaction. The Se-dependent isoform, also referred to as Se-dependent GPx, consists of four subunits, and each subunit contains a Se atom in the active site bound to the amino acid cysteine. Except for mammalian GPx 5 and 6, all glutathione peroxidases are selenoproteins and contain a SeCys residue instead of Cys as part of their active site.

The highest GPx activity is detected in the liver, while medium activity is observed in the heart and lungs [42]. GPx enzyme cooperates with reduced glutathione GSH and is present in cells at high concentrations (millimoles). The GPx enzyme degrades peroxides to water or alcohol, while GSH is oxidised, reducing glutathione Se from the catalytic centre of the enzyme, Figure 15. Selenol ($-\text{SeH}$) in GPx, which contains selenocysteine Sec, reacts as selenolate with H_2O_2 to form seleninic acid $-\text{SeOH}$, which is reduced by two molecules of GSH back to $-\text{SeH}$, forming GSSG and water [43]:

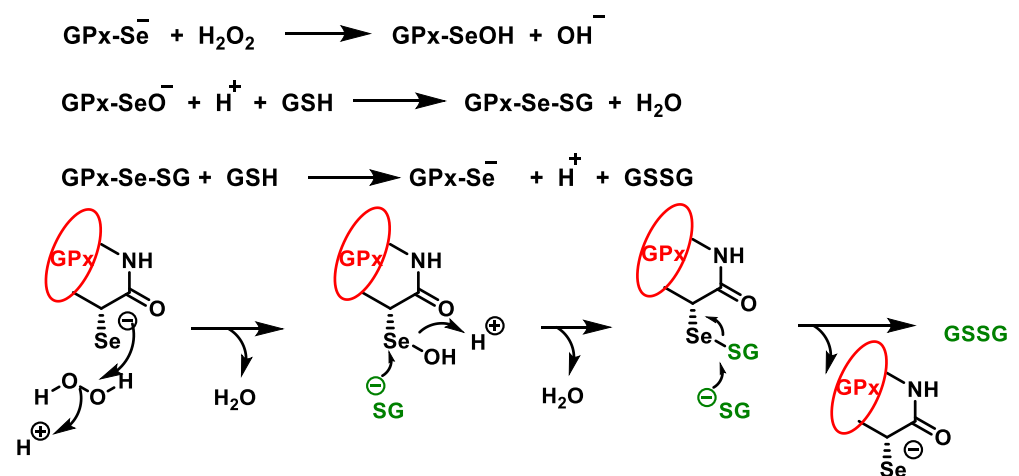


Figure 15. Catalytic cycle of glutathione peroxidase for the reduction of H_2O_2 .

In the peroxidative part of the cycle, the selenol in GPx is oxidised by hydrogen peroxide to seleninic acid. The first GSH molecule forms a selenium disulphide with the seleninic acid, with the oxygen leaving as a water molecule. The second GSH molecule reduces the selenium disulphide by thiol-disulphide exchange, the GSSG is released, and the enzyme is regenerated to its selenol form to begin a new cycle.

The GSSG produced by the GPx enzyme in this process, as well as in other metabolic processes, must be constantly recycled in the cell for the peroxidative system to function properly. This is done by glutathione reductase GR, which is responsible for reducing GSSG to GSH. This disulphide bridge is reduced using NADPH as an electron source, Figure 16.

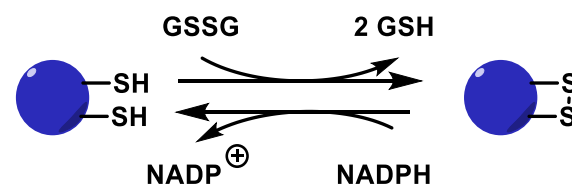


Figure 16. Glutathione reductase catalytic cycle.

3.3. Peroxiredoxins (Prx) and Thioredoxins (Trx)

Prx are a large family of antioxidant enzymes capable of breaking down peroxides that have cysteine in their active center [44]. These enzymes are responsible for controlling peroxide levels and are found in the cytosol, nucleus, membranes, mitochondria, Golgi complex, peroxisomes, and extracellular fluids. Prxs form a family of enzymes that, depending on the isoform or species, can detoxify hydrogen peroxides, peroxides of long-chain organic compounds, phospholipids and fatty acids, and peroxyntirite [45].

In the case of peroxiredoxin containing a cysteine (1-Cys-Prx or Prx6 isoform), the SOH formed during peroxide reduction is not attacked by the cysteine enzyme but is reduced using glutathione GSH as an electron source and forming glutathione disulphide GSSG, a mechanism like that of glutathione peroxidase. Reduction of SOH (1-Cys-Prx) or S-S (typical and atypical 2-Cys-Prx) allows regeneration of the active forms of Prx, Figure 17, left. Except for the Prx6 isoform, these enzymes contain two cysteines in the active site. One is a thiol that is oxidised to sulphenic acid SOH during the catalytic cycle. The other is a resolving cysteine that attacks SOH by forming an intermolecular S-S (typical 2-Cys-Prx or Prx1–Prx4 isoforms) or intramolecular (atypical 2-Cys-Prx or Prx5 isoform) disulphide bridge. The disulphide bridge formed in the catalytic cycle is reduced using reduced thioredoxin Trx_{red} as an electron source to generate oxidised thioredoxin Trx_{ox} , Figure 17, right.

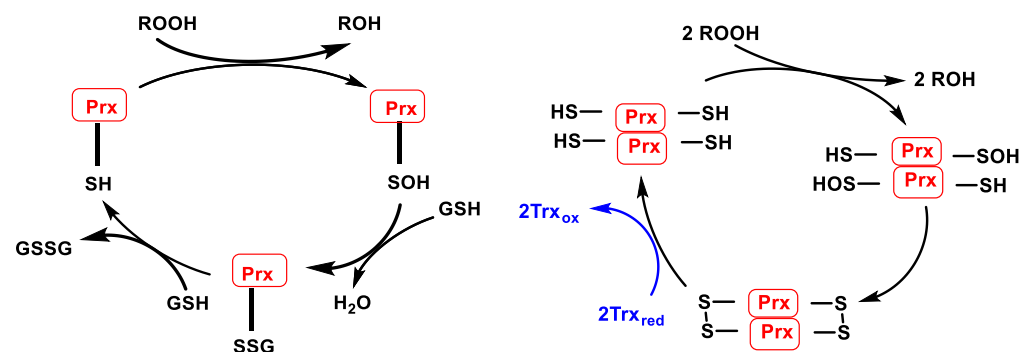


Figure 17. (Left): Catalytic cycle of Prx containing 1 catalytic cysteine (1-Cys-Prx). (Right): Catalytic cycle of typical Prx (2-Cys-Prx).

Atypical peroxiredoxins (atypical 2-Cys-Prx) are monomers with two cysteines participating in the catalytic cycle in a single subunit. In all cases, one cysteine SH is oxidised to sulphenic acid SOH by reducing peroxides ROOH. In the atypical 2-Cys-Prx, SOH is reduced by a cysteine present in the same subunit, resulting in an intramolecular disulphide bond that is also reduced by Trx_{red} , Figure 18.

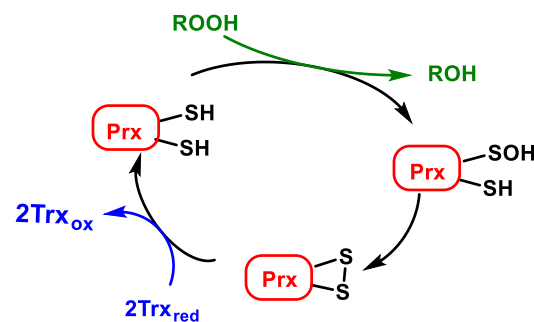


Figure 18. Catalytic cycle of atypical peroxiredoxins (atypical 2-Cys-Prx).

Trx are proteins that act as antioxidants by facilitating the reduction of other proteins through thiol-disulphide exchange on cysteine. Thioredoxin reductase TrxR is responsible for the reduction of Trx_{ox} to Trx_{red} and requires NADPH as an electron donor source [46], Figure 19.

When used as an electron source in the catalytic peroxiredoxin cycle, thioredoxin Trx meets the thiol group SH of its two cysteines in disulphide form S-S. TrxR, which has a selenocysteine in selenol form SeSH and a SH at its catalytic site, performs the S-S reduction of Trx, forming an intermolecular sulphur-selenium bridge S-Se. This bridge is then reduced, creating an intramolecular S-Se bridge in TrxR and releasing the Trx in reduced form. TrxR regenerates into its reduced form using NADPH as an electron source [47].

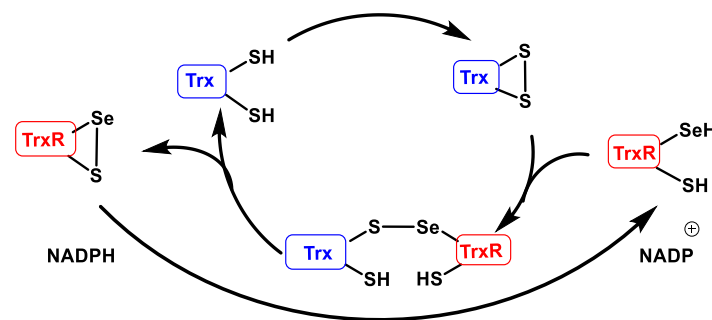


Figure 19. Thioredoxin reductase catalytic cycle (TrxR).

3.4. Hypochlorous Acid Formation (HOCl)

HOCl biologically falls into a group of small molecules known as reactive species RS synthesised by cells of the immune system (neutrophils and macrophages). HOCl plays a dual role, performing a bactericidal function against infections and, on the other hand, it can cause damage to the molecular structures and cells of the host organism. HOCl is a powerful antimicrobial oxidant, capable of modifying DNA, lipids and lipoproteins, reacting rapidly with the sulphur atom present in thiols and thioethers (cysteine and methionine) [48]. Excessive generation of HOCl can cause tissue damage and is thought to be important in the progression of a number of diseases including atherosclerosis, chronic inflammation and some cancers [49].

Myeloperoxidase is the most abundant protein in neutrophils and is the only peroxidase that catalyses the conversion of hydrogen peroxide and chloride to hypochlorous acid [50], Figure 20.

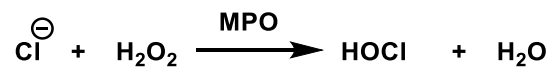


Figure 20. Conversion of hydrogen peroxide and chloride to hypochlorous acid.

When the human body is invaded by bacteria or viruses, our immune system responds immediately by sending an increased number of white blood cells called neutrophils to the site of invasion. Once activated, neutrophils produce through the enzyme myeloperoxidase large quantities of an oxidising solution (HOCl), which is highly effective in killing all pathogenic invading microbes.

4. H₂O₂ and Inflammation

Inflamed tissues are associated with high levels of H₂O₂, and this mechanism plays an important role in host antimicrobial defence [51]. During inflammation, H₂O₂ by-products interact with proteins, lipids, nucleic acids and other metabolites causing associated molecular damage that can be significant and lead to cell apoptosis [52]. Inflammation, which is predominantly caused by activated macrophages and microglia, is a contributing factor to some chronic diseases, such as Alzheimer's disease [53,54].

A likely mechanism describing the sequence of events is as follows: (a) hydrogen peroxide is produced in reaction to a pro-inflammatory ligand, e.g., by NADPH oxidase and (b) hydrogen peroxide diffuses across cell membranes into adjacent target cells [55].

Both H₂O₂ and TNF- α (tumour necrosis factor alpha is a cytokine produced by various cells of the immune system, mainly macrophages and monocytes) are formed during inflammation, but H₂O₂ has little ability to activate NF- κ B (nuclear enhancer factor kappa light chain enhancer of activated B cells is a protein complex that controls DNA transcription), so H₂O₂ up-modulates TNF- α and this induces the NF- κ B activation [56]. NF- κ B is involved in inflammation, innate and adaptive immune responses to viral infection, cell proliferation and apoptosis [57].

To avoid excessive tissue damage and sustained granulocyte recruitment/retention, the presence of the H₂O₂ gradient must be tightly regulated at the enzymatic level. Oxidase

enzyme activity causes membrane depolarization to the point of inhibition of NADPH oxidase. Sustained H_2O_2 production depletes NADPH stores, which may automatically lead to cessation of H_2O_2 production [51,58].

5. H_2O_2 and Cancer

As a result of increased metabolic activity, high rates of H_2O_2 are generally detected in neoplastic cells, where they act as signalling molecules in tumour development and even progression. At the same time, these cells are able to express higher levels of antioxidant proteins to attenuate their effect, creating a balance between the continued generation of H_2O_2 and antioxidant molecules [14]. Certain doses of H_2O_2 and superoxide anions stimulate cell proliferation in a variety of cancer cell types [59]. This is the case in breast cancer cells, where H_2O_2 is increased through translocation of oestrogen to the mitochondria [60]. An increase in mitochondrial oxidative stress causes the release of cytochrome C, which in turn is an irreversible event, leading to caspase activation and cell death [61]. All human cancer cell types, except human renal adenocarcinoma, have shown low levels of catalase and glutathione peroxidase [62]. In general, catalase concentration is low in cancer cells, but its activity seems to vary widely between different cancer cell lines [63].

For an in-depth review of this topic, we have chosen the oxidative mechanism in leukaemia as an explanatory model, because it is a pathology that affects pluripotential stem cells, generating systemic involvement. Severe or extended oxidative stress OS inevitably destroys their homeostasis, affecting the self-renewal and differentiation of hematopoietic stem cells HSCs and leading to hematopoietic abnormalities. Balanced OS levels are crucial for maintaining the homeostasis and biological function of HSCs under normal conditions [64].

In normal HSCs, overactivation of oxidative stress pathways promotes the production and intracellular accumulation of $\bullet O_2^-$ and H_2O_2 , severely disturbs the normal biological functions of HSCs and has an important role in leukaemia progression [65,66].

In summary, as active secondary signalling molecules, OS have important inductive and regulatory roles at various stages of ALL development and progression. Defects in antioxidant defence systems might promote the production of intracellular accumulation of OS, which may seriously disrupt the normal biological functions of hematopoietic cells. and induce genetic lesions considered determinant and crucial for leukemic transformation of normal HSCs and/or hematopoietic progenitors, leading to the development of leukaemia. The mechanism of action of OS on proteins and lipids at the molecular level is basically clear. An inadequate understanding of redox signalling in normal and malignant HSCs severely limits the efficacy of all treatment. Drug resistance and side effects caused by pro-oxidant drugs remain an urgent and difficult problem in the treatment of leukaemia [67].

6. H_2O_2 and Related Diseases

Among the diseases associated with ROS, ischemia-reperfusion I/R injury is one of the best studied, along with myocardial infarction, stroke, and other thrombotic events. Reperfusion occurs when blood circulation is restored to tissues after ischemia, as the restoration of circulation after the absence of nutrients and oxygen triggers inflammation, and the subsequent oxidative stress damages affected tissues [68]. In an attempt to determine the relationship between H_2O_2 and I/R injury, its accelerated production in postischemic tissues was observed to be caused by enzymes capable of reducing molecular oxygen to superoxide anions, thereby releasing H_2O_2 into the extracellular and intracellular space [69]. After reperfusion of ischemic tissue, the production of superoxide anions, which have higher levels than the nitric oxide radical NO, was favoured, so that endothelial cells produce more superoxide anion molecules and the production of NO by endothelial NO synthase eNOS decreases [70].

Ulcerative colitis is a type of inflammatory bowel disease that strikes between late adolescence and early adulthood [71] and follows a chronic relapsing and remitting course characterised by abdominal pain, bloody diarrhoea, tenesmus and urgency, all related to

inflammation of the large intestine [72]. An immune abnormality is a possible mechanism that may cause this disease, although it has not been established as a primary antecedent in individuals with UC or their family members [73,74]. In contrast, significantly elevated levels of H_2O_2 have been documented in the colonic epithelium immediately prior to the onset of UC, suggesting a causal role in the development of UC [75].

Sepsis is a life-threatening condition triggered as an extreme response to infection that can lead to multiorgan failure and fatal hemodynamic shock. During the course of sepsis, cytotoxic hydrogen peroxide levels in the blood have been documented to be up to 18 times higher than the accepted limit for normal blood levels in individuals with sepsis and septic shock [76]. The normal blood value of H_2O_2 is between 1 and 5 μM , and the value at which cytotoxicity appears is 30 μM [77]. Despite this, values of up to 558 μM have been documented in the blood of patients with sepsis and septic shock [76]. Hypermetabolism is a feature of sepsis, with increased metabolic activity supplied by ATP generated by oxidative phosphorylation, producing H_2O_2 as a by-product of the electron transport chain (ETC). Under normal conditions, this H_2O_2 is effectively neutralized, but an increase in bioenergetic reactions in the ETC leads to an increase in the production of hydrogen peroxide, which, if not removed, can induce cell death. Cytotoxic levels of hydrogen peroxide are a metabolic poison that can lead to severe bioenergetic dysfunction and cellular damage if a regulatory mechanism is not employed. Prolonged exposure can lead to collapse of redox homeostasis, organ failure, microvascular dysfunction, and fatal septic shock [78].

H_2O_2 inhibits the functioning of Krebs cycle enzymes, such as aconitase, alpha-ketoglutarate dehydrogenase, and succinate dehydrogenase [79–81], decreasing production of the reducing equivalents nicotinic adenine dinucleotide NADH and flavin adenine dinucleotide FADH₂, both of which are involved in cellular redox reactions. This can collapse the mitochondrial proton gradient and affect the proton motive force required for pyruvate translocase in the inner mitochondrial membrane and transport pyruvate into the mitochondria [82]. The result is an increase in cytosolic pyruvate and subsequent conversion to lactate with the onset of hyperlactatemia. The effect of a dysfunctional Krebs cycle on the serum lactate level is seen in hereditary alpha-ketoglutarate dehydrogenase deficiency, associated with severe congenital hyperlactatemia [83].

In the pancreas, β -cells are particularly vulnerable to oxidative stress due to a relatively reduced expression of antioxidant enzymes, such as catalase, superoxide dismutase and GSH peroxidase, compared to levels in the liver and kidney [84–86]. It has been hypothesized that H_2O_2 are involved in the progression of cell dysfunction in both type 1 and type 2 diabetes mellitus. Diabetes induces increased levels of hydrogen peroxide [87,88], a metabolic disorder characterised by hyperglycaemia and often associated with the occurrence of complications. The detoxification pathway utilises the antioxidant peroxiredoxin/thioredoxin system as it provides selective chemical inhibition. The rate of mitochondrial oxidation in pancreatic β -cells is directly dependent on blood glucose concentration. In type 2 diabetes, where blood glucose levels are chronically elevated, increased oxidative phosphorylation leads to increased H_2O_2 production in the β -cells [89]. Overexpression of the SOD1 and SOD2, catalase, GSH peroxidase or thioredoxin in β -cells offers protection against oxidative damage induced by alloxan, the combination of xanthine oxidase and hypoxanthine, streptozotocin or H_2O_2 [90–92]. Recent studies have shown that peroxiredoxins, an antioxidant enzyme capable of reducing hydrogen peroxide, lipid peroxides, and peroxynitrite, are expressed in β -cells and, when overexpressed, protect β -cells from oxidative stress [93,94].

The following Table 1 summarizes the relationship between H_2O_2 and disease (including recent research findings).

Table 1. Relationship between H₂O₂ and disease.

Pathology	Levels of H ₂ O ₂	Location	Mechanism
Cancer	Plasma increases 2–3 times higher than normal	Mitochondrial membrane of neoplastic cells	Stimulation of cell proliferation by increased metabolic activity [95]
Ischaemia-reperfusion injury (I/R)	Plasma levels higher than NO concentration	Extra- and intracellular spaces	Oxidative stress secondary to tissue damage [96]
Sepsis and septic shock	Plasma increases 18 times higher than normal	Endothelial cells	Increased oxidative phosphorylation by metabolic hyperdemand [97]
Ulcerative colitis	Significantly increased urinary excretion levels	Colon epithelium	Mechanism under study (not known at present, also implicated in other autoimmune diseases), although a causative role is suggested [98]
COVID-19 and respiratory distress syndrome RDS	Very high plasma levels especially in combination with urinary sepsis	Endothelial cells mainly from the lung	Expansion of ACE 2 protein leading to increased cellular oxidative status [99,100]
Intestinal parasitic infection	Urinary excretion 4 times higher	Intestinal endothelial cells	Oxidative stress secondary to phagocytosis [101,102]
Diabetes mellitus type II	3-fold increase in superoxide dismutase with associated decrease in erythrocyte catalase leading to increases in peroxide excretion.	Pancreatic beta cells	Increased oxidative Phosphorylation [103,104]

7. Measurement of Hydrogen Peroxide in Human Body

Currently, there is considerable interest in developing “biomarkers” of oxidative stress that can be applied to humans. These involve measuring the end products of oxidative damage in different classes of biomolecules or directly determining the production of oxygen radicals. H₂O₂, present in human body fluids, is a valuable biomarker of oxidative stress in vivo and can be detected in exhaled air, urine and blood [105].

7.1. Presence in Blood

Blood H₂O₂ levels are of great importance, but rapid and reliable measurement remains a challenge. Gaikwad et al. (2021) present an automated method using a microfluidic device for direct and rapid measurement of H₂O₂ based on laser-induced fluorescence measurement and explain the critical factors affecting measurement accuracy: Blood cells and soluble proteins significantly alter the native H₂O₂ content in the time between sample collection and detection. Separation of the blood cells and subsequent dilution of the plasma with a buffer allow reliable measurements. The method allows rapid measurement of H₂O₂ in plasma in the concentration range of 0–49 μM, with a detection limit of 0.05 μM, sensitivity of 0.60 μM⁻¹, and detection time of 15 min, achieving real-time control [106].

7.2. Presence in Urine

Hydrogen peroxide can be detected in sick and healthy individuals, as there is a correlation between urinary H₂O₂ levels and other biomarkers of oxidative stress. Urinary H₂O₂ is a useful biomarker for assessing oxidative status in humans and for predicting disease pathogenesis and progression. Several methods for measuring urinary H₂O₂ are described in the scientific and technical literature, and the FOX assay is a widely used method for its determination. The FOX assay involves the oxidation of Fe²⁺ to Fe³⁺ by H₂O₂ and the subsequent formation of a chromophore (Fe³⁺-xylene orange complex) that can be measured at 560 nm. Previous studies on the detection of H₂O₂ in urine have mainly used the FOX –2 assay, which is usually used to measure the concentration of hydroperoxide in plasma and seems to be unsuitable for urine samples, whereas a pH-adjusted FOX –1

assay (pH 1.7–1.8) in the presence of catalase has been proposed as a method with high sensitivity and specificity for the detection of H₂O₂ in urine [107].

Lipsey et al. (2022) analysed urinary H₂O₂ concentrations in 82 patients with severe infections (sepsis, septic shock, and infections that did not meet sepsis 3 criteria), patients with severe burns and associated systemic inflammation, and healthy volunteers and found higher concentrations in patients who died within 28 days of ICU admission than in patients who survived. This finding was also consistent in subgroups of patients with severe infections and burns, suggesting that H₂O₂ is associated with a poor prognosis in these patients [97].

7.3. Presence in the Exhaled Air

Inflammatory lung processes may be associated with oxidative stress. The degree of oxidative stress can be determined by measuring the concentration of hydrogen peroxide in exhaled breath condensate, an easily collected, non-invasive, and affordable tool for diagnosing the inflammatory process. Exhaled breath condensate (EBC) contains a wide range of inflammatory mediators, oxidative stress, and nitrosative stress, the analysis of which can aid in the diagnosis and treatment of patients with lung disease [108].

Different types of equipment are available on the market, including commercial capacitors and home-made systems. Among the different devices, the following can be highlighted: EcoScreen1 (Cardinal Health, Hoechber, Germany, currently not manufactured), EcoScreen2 (FILT Lungen-& Thorax Diagnostik GmbH, Berlin, Germany), RTube (Respiratory Research, Austin, TX, USA), TurboDECCS (Medivac, Parma, Italy), ANACON (Biostec, Valencia, Spain). Samples for the evaluation of non-volatile compounds should be stored immediately after collection at −80 °C until analysis [109].

H₂O₂ concentrations in the EBC were increased in steroid-free asthma and were influenced by smoking and disease treatment [110]. H₂O₂ levels were elevated and pH was lower in both asthma and chronic obstructive pulmonary disease COPD compared to control subjects. These findings suggest that oxidative stress is involved in the pathogenesis of asthma and COPD and that H₂O₂ levels in the EBC may reflect health status in COPD [111].

8. Conclusions

In this review, the chemical role of H₂O₂ in mammalian cells and its enzymatic and non-enzymatic generation and regulation were studied in detail. Cells have multiple sources of H₂O₂, but also scavenger molecules that tightly control H₂O₂ concentration in different subcellular compartments. To better understand the molecules and targets involved in this delicate balance (physiological/pathological H₂O₂ concentration), it is important to know in detail the molecular mechanisms operating at the cellular level. We hope that our work will stimulate the scientific community to better understand these important events and processes.

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