Phagocyte and extra-phagocyte myeloperoxidase-mediated microbicidal action

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Myeloperoxidase (MPO) is produced by neutrophil leukocytes and monocytes. These leukocytes are the phagocyte effectors of the acute inflammatory response and provide innate protection against infecting pathogens. In that regard MPO is the enzymatic effector of phagocyte microbicidal action. MPO is unique in its ability to catalyze the hydrogen peroxide (H_2O_2) dependent oxidation of chloride (Cl^-) to hypochlorite (OCl^-), i.e., the microbicidal component of bleach. Hypochlorite directly reacts with a second H_2O_2 to produce singlet molecular oxygen ($^1O_2$*), an electronically excited state of oxygen with potent electrophilic reactivity. As a metastable excited state, $^1O_2$* has a microsecond half-life that restricts microbicidal reactivity to within a radius of about 0.2 micron from its point of generation. In unstimulated phagocytes, MPO is stored in the azurophilic granules. Following the phagocytosis of a microbe, azurophilic granules fuse with the microbe-containing phagosome to form a phagolysosome. In the process of phagocytosis NADPH oxidase is activated and incorporated into the membrane of the phagosome, and ultimately, the membrane of the phagolysosome. NADPH oxidase catalyzes the one-equivalent reduction of oxygen (O_2) producing hydrodioxylic acid (HO_2; aka, hydroperoxyl radical) with an acid dissociation constant (pK_a) of 4.8. As such, production of HO_2 drives the pH of the phagolysosomal space toward 4.8. At neutral pH, HO_2 dissociates yielding a proton (H^+) and its conjugate base, the superoxide anion (O_2^-). Although anionic charge repulsion prevents direct disproportionation of O_2^- in neutral to alkanine conditions, in an acid milieu HO_2 readily reacts with O_2^- yielding the H_2O_2 that drives MPO production of the OCl^- and $^1O_2$* necessary for effective microbicidal action. Healthy human adults release about 10^{11} neutrophils containing about 0.4 mg MPO into the circulating blood each day; i.e., about 4 femtograms MPO/neutrophil. The production of neutrophils and the quantity of MPO per neutrophil increase in inflammatory states and with G-CSF treatment. After a relatively short circulating lifetime, neutrophils leave the blood and migrate into body spaces including the mouth, urinary tract, vagina and gastrointestinal tract. Greater than 10^5 neutrophils can be routinely lavaged from the mouth of healthy humans, and in inflammatory conditions, the quantity of neutrophils lavaged increases in proportion to the blood neutrophil count. The oropharyngeal and vaginal spaces are normally colonized with lactic acid bacteria (LAB), such as viridans streptococci and lactobacilli. These LAB lack cytochromes and produce lactic acid and H_2O_2 as end products of metabolism. Consequently, the neutrophil leukocytes that migrate into such spaces can release MPO into an acidic milieu containing H_2O_2 adequate to facilitate extra-phagocyte microbicidal action. MPO selectively binds to microbes, showing relatively low binding to LAB and high binding to other bacteria. The short half-life of $^1O_2$* demands proximity to target for effective killing, and as such, when MPO is limiting, selective binding results in selective killing. The fundamental elements required for phagolysomal and extracellular microbicidal action are MPO, Cl^- and H_2O_2 in an acidic milieu. As such, combining purified MPO and a H_2O_2-generating oxidase with oxidase substrate results in a potent microbicidal system. In that regard, effective and broad spectrum MPO microbicidal action has been demonstrated using glucose oxidase (GO) with glucose as substrate. This GO-MPO system demonstrates *in vitro* microbicidal action against all bacteria and yeast tested. The efficacy of applying this GO-MPO microbicidal formulation for the prevention of surgical site and wound infections has also been demonstrated in animal test models.

Keywords myeloperoxidase, hydrogen peroxide, hypochlorite, singlet oxygen, phagolysosome, oxidase, selective binding, streptococci, lactic acid bacteria, microbicidal

1. Oxygen-dependent microbicidal action of phagocytic leukocytes

Neutrophil leukocytes and monocytes are the phagocyte effectors of the acute inflammatory response that provide innate protection against infecting pathogens [1]. Hematopoietic bone marrow has a mass of about 2.5 kg in healthy human adults, and greater than fifty percent of this hematopoietic activity is dedicated to synthesis of these phagocytic leukocytes [2].

Differentiation of blasts to promyelocytes is the first step in the maturation path to neutrophils and monocytes. Development within the promyelocyte pool is characterized by synthesis of azurophilic (aka, primary) granules containing cationic proteins, especially myeloperoxidase (MPO). MPO, a 145 kDa dimeric alpha-heme haloperoxidase present in azurophilic granules, makes up approximately 5% of the dry weight of the neutrophil [3-4]. Assuming that a human neutrophil has a cell volume of about 450 femtoliter (fL), a specific gravity of 1.1, and a cell water content of 84 %, the MPO content per neutrophil is estimated to be about 4 femtograms (fg). In addition to this high MPO content, azurophilic granules contain other cationic proteins and hydrolytic enzymes, and as such azurophilic granules are lysosomal in nature.

The cessation of azurophilic granule synthesis marks the end of promyelocytic development, and signals the beginning of the myelocytic stage of development. No MPO is synthesised during myelocyte development, and thus,

each cell division within the myelocyte pool decreases the azurophilic granules and MPO content of the resulting mature neutrophil by one half.

Development within the myelocyte pool is characterized by the synthesis of specific (aka, secondary) granules. These specific granules contain components necessary for integrin (i.e., CD11b) and NADPH oxidase (i.e., gp91(*phox*)) function [5]. Relative to azurophilic granules, specific granules are small in size with a high proportion of membrane to granule content. Specific granules provide the reserve membrane necessary for phagocyte chemotaxis and phagocytosis. The myelocyte pool is the last stage of neutrophil development where the cell is capable of division. Although less mature than segmented neutrophils, metamyelocytes and band neutrophils are capable of phagocytosis and microbicidal action.

About a hundred billion (10^{11}) neutrophils are released into the circulating blood each day. After a relatively short time within the circulation, i.e., <10 hrs, neutrophils leave the blood to enter the tissue pool and body spaces [2]. Inflammatory states or treatment with cytokine colony stimulating factors, i.e., human recombinant G-CSF and GM-CSF, increase production of neutrophils and monocytes as much as tenfold [6]. From the opposite perspective, severe neutropenia, i.e., neutrophil blood counts <500/μL, is associated with high susceptibility to infection. The critical role of phagocytic leukocytes in host defence against pathogenic microbes is emphasized by the magnitude of neutrophil production under normal conditions, the large increase in production in response to inflammation, and increased host susceptibility to infection associated with neutropenia, i.e., an inability to meet neutrophil demand.

1.1 Specific degranulation, phagocytosis, and activation of respiratory burst metabolism

Providing innate protection against infecting pathogens requires that the phagocyte: (1) interact and communicate with the endothelial cells that line the vasculature, (2) respond to inflammatory cytokines, (2) undergo diapedesis through the endothelium into the interstitial tissue space, (3) move through the tissue (chemotaxis) to the site of infection, (4) recognize opsonised microbes on contact, and (5) phagocytose the microbes.

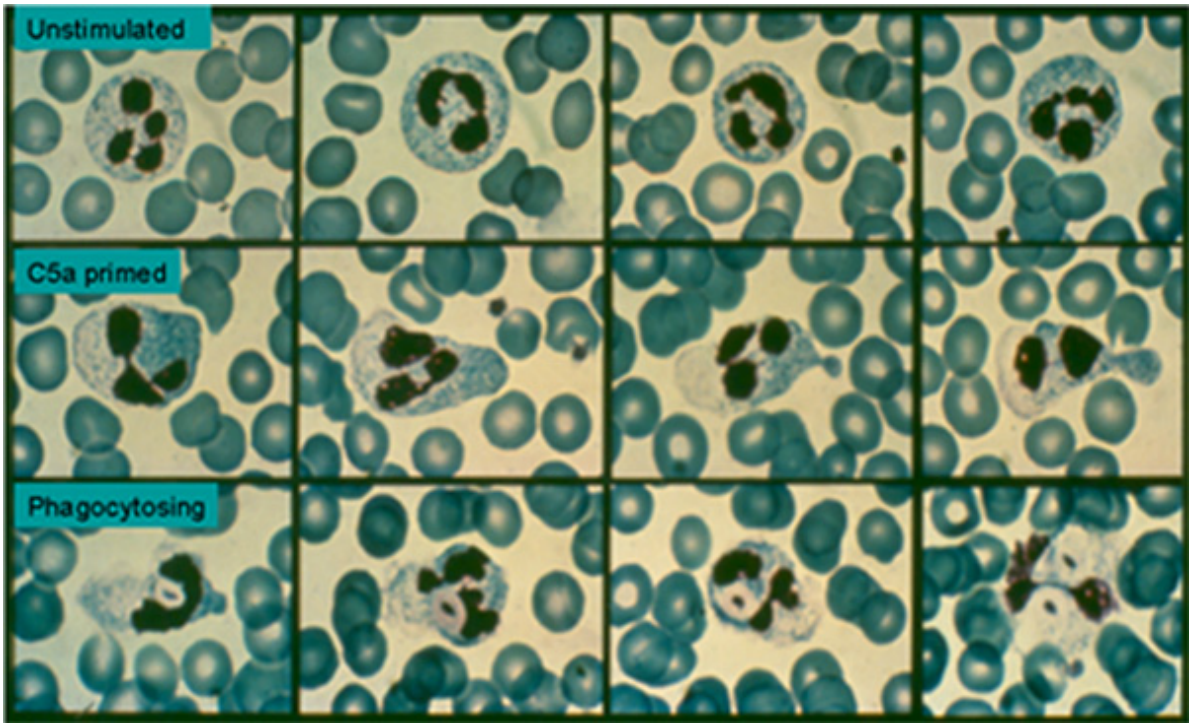

Fig. 1 Blood smear neutrophil leukocyte showing morphologic changes associated with degranulation and phagocytosis. The top four frames show normal unstimulated neutrophils in a background of erythrocytes. The middle four frames show the morphologic consequence of neutrophil specific degranulation in response to the anaphylatoxin C5a. The frames from left-to-right show the progression of "polar" morphologic changes from the early-to-late stages of specific degranulation. The bottom four frames show neutrophils phagocytosing complement (C3bi) opsonified zymosan. The frames from left-to-right show the progression of phagocytosis with fusion of azurophilic granules to generate the MPO-rich phagolysosome.

In the unstimulated state, neutrophils have spherical morphology, as illustrated by the neutrophils shown in the top frames of Figure 1. Spherical morphology guarantees minimum surface to volume. Specific degranulation, i.e., fusion of the specific granules with the neutrophil surface membrane, provides additional surface membrane. Specific degranulation is also required for membrane assembly of integrin (CD11b) and opsonin receptors (CR 1; CD35), cytokine/anaphylatoxin (C5a) receptors, and assembly and activation of NADPH oxidase [5, 7].

The morphologic changes associated with specific degranulation and membrane fusion are shown in the middle frames of Figure 1. The more subtle early specific degranulation changes are shown by the neutrophils in the frames on the left. The "polar" morphologic changes of the neutrophils in the frames on the right indicate more complete degranulation [8]. Degranulation provides membrane necessary for chemotaxis and ultimately phagocytosis. The phagocytic process is a membrane invagination, i.e., the outer membrane contacting the microbe binds to and ultimately envelopes the microbe into a phagosome. The exterior membrane of the neutrophil becomes the exterior membrane of the phagosome that retains its association with the microbe; the interior membrane continues its association with the neutrophil cytoplasm. Phagocytosis consumes surface membrane and increases cell volume; as such, increase in surface membrane is physically required for phagocytosis.

After engulfment and suspension within the cytoplasm, the phagosome fuses with the azurophilic granules that are rich in MPO and other cationic proteins. The resulting phagolysosome is the apparatus of optimal microbicidal action.

1.2 Respiratory burst metabolism, phagolysosome formation, and myeloperoxidase (MPO)

The morphologic changes of phagocytosis are associated with impressive biochemical changes. These metabolic changes, often collectively referred to as "respiratory burst" metabolism, are characterized by a large increase in glucose metabolism via the dehydrogenases of the hexose monophosphate shunt (aka, pentose pathway), and an equally large increase in mitochondria-independent oxygen (O_2) consumption [9-10].

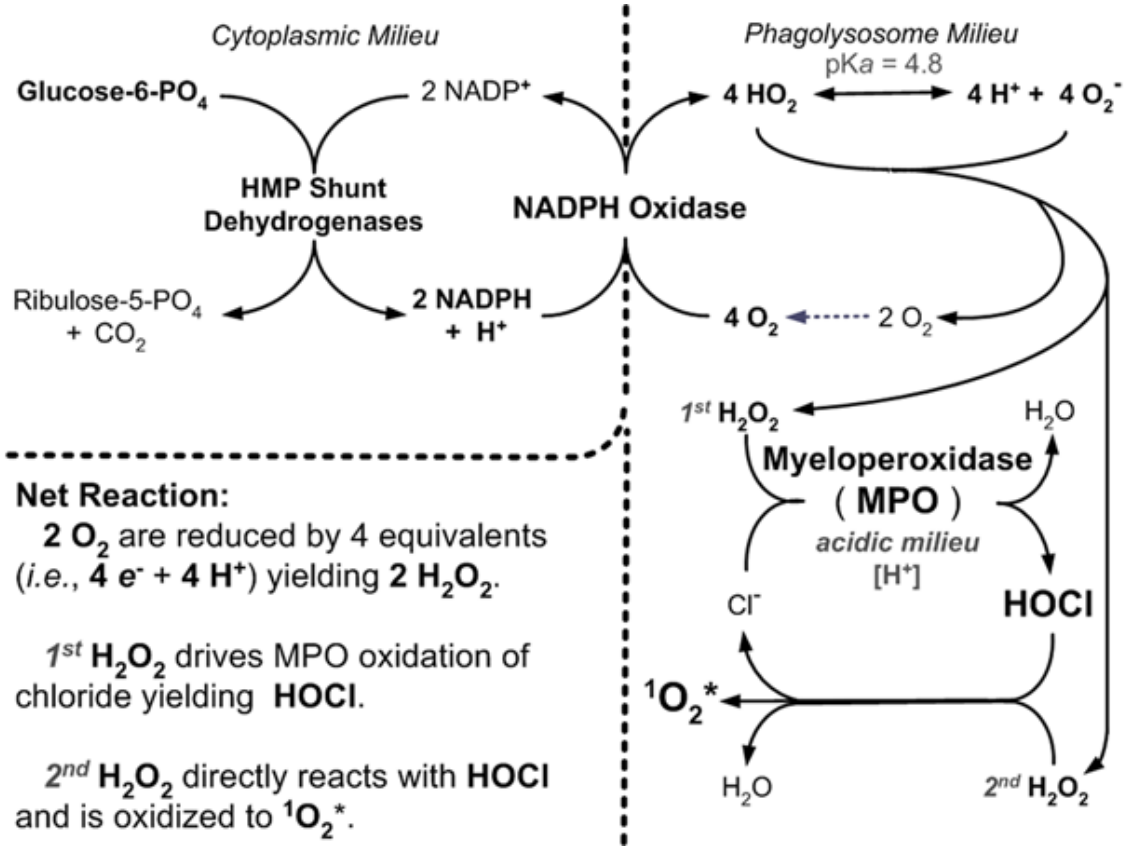

Fig. 2 Schematic depiction of NADPH oxidase-driven MPO oxidation of chloride to hypochlorous acid (HOCl), and hypochlorite oxidation of hydrogen peroxide (H_2O_2) producing electronically excited singlet molecular oxygen ($^1O_2$*) [10].

Phagocytosis is linked to the activation of NADPH oxidase that catalyzes the univalent reduction of O_2 using reducing equivalents supplied by NADPH [10]. The resulting oxidized $NADP^+$ is the rate-limiting substrate for glucose oxidation via the dehydrogenases of the hexose monophosphate (HMP) shunt (aka, pentose pathway). An activated neutrophil metabolizes $4.7\pm0.5x10^{10}$ glucose molecules/neutrophil/h through the glycolytic pathway. Of this, $1.5\pm0.6x10^{10}$ glucose molecules/neutrophil/h are metabolized through the dehydrogenases of the HMP shunt, and thus, about 3.0×10^{10} NADPH molecules/neutrophil/60 min, i.e., $6.0x10^{10}$ reducing equivalents/neutrophil/h [11-12].

As illustrated in the schema of Figure 2, NADPH oxidase applies the equivalents of NADPH to univalent reduction of oxygen (O_2) producing hydrodioxylic acid (HO_2; aka, hydroperoxyl radical). The acid dissociation constant (pK_a) of HO_2 is 4.8, and as such, HO_2 dissociates yielding a proton (H^+) and its conjugate base, superoxide anion (O_2^-), at neutral pH. Production of HO_2 acidifies the phagosomal and phagolysosomal space [13]. Although anionic charge repulsion

prevents direct disproportionation of O_2^- in neutral to alkaline conditions, HO_2 readily reacts with O_2^- yielding one H_2O_2 and one O_2 in acid milieu. Approximating glucose metabolism, H_2O_2 production is reported to be $5.8\pm1.0x10^{10}$ H_2O_2 molecules/neutrophil/h [14].

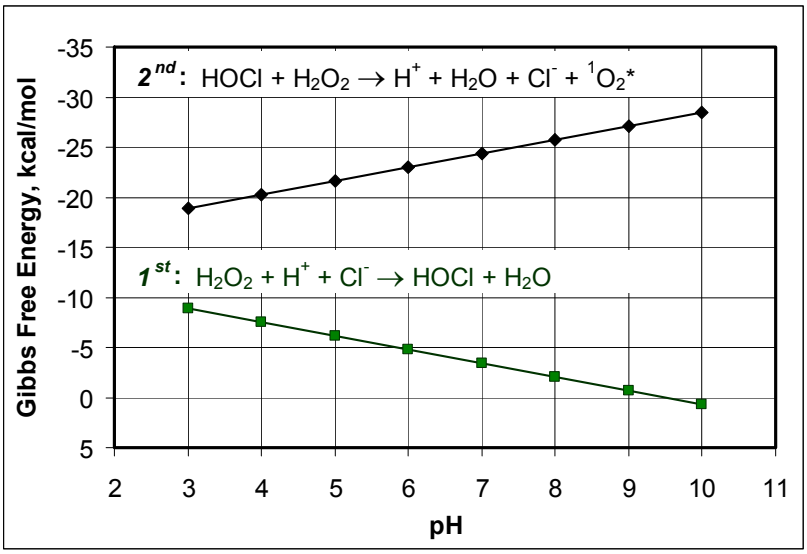

Fig. 3 The MPO-dependent oxidation of chloride to hypochlorous acid (1st Reaction) and the oxidation of H_2O_2 to $^1O_2$* (2nd Reaction) are presented with regard to Gibbs free energy of the reactions over a range of pH. The Gibbs free energy for the 2nd Reaction is adjusted for the energy of $^1O_2$*, i.e., 22.5 kcal/mol.

For each molecule of glucose oxidized, two H_2O_2 molecules are ultimately released into the acidic phagolysosomal space containing abundant MPO. The first H_2O_2 is substrate for MPO haloperoxidase action. Of the mammalian peroxidases, MPO is unique in its ability to catalyze the two electron oxidation of chloride (Cl^-) producing hypochloric acid (HOCl) [15-16]. As illustrated by the plot of Gibbs free energy against pH shown in Figure 3, pH significantly influences the energy yield of these electrochemical reactions [17]. The relationship of electrochemical potential to Gibbs free energy is $\Delta G^o = -nF\Delta E^o$, where ΔG^o is the standard change in Gibbs free energy, n is the number of electrons per gram equivalent transferred, F is the Faraday constant, and ΔE^o is the standard change in potential. A reaction is exergonic when energy is set free, i.e., when ΔE^o is positive and ΔG^o is negative. The MPO-catalyzed oxidation of Cl^- to HOCl (1st Reaction) is exergonic in acid milieux. Note that exergonicity decreases with increasing pH, and that the reaction is endergonic, i.e., energy requiring, at pH above 9.5.

The oxidation of H_2O_2 to $^1O_2$* (2nd Reaction) is highly exergonic. Note that the relationship of pH to Gibbs free energy is opposite to that observed for the 1st reaction; i.e., the reaction becomes more exergonic with increasing pH. At any given pH, the total Gibbs free energy for the combined reactions equals -27.8 kcal/mol. This value includes the downward adjustment in free energy, i.e., 22.5 kcal/mol, required to balance $^1O_2$* generation.

1.3 MPO-mediated combustive microbicidal action and chemiluminescence

The microbicidal action of HOCl is common knowledge. It is a reactive oxidizing agent capable of dehydrogenating organic molecules and directly reacting with amines to produce chloramines. The reactivity of singlet molecular oxygen is dramatically different from that of triplet multiplicity ground state oxygen. Understanding the potent reactivity of $^1O_2$* is best approached through a consideration of the Wigner spin conservation rules [18-21]. Reaction of $^1O_2$* with singlet multiplicity bioorganic molecules is spin-allowed and combustive in nature, i.e., oxygen is incorporated into the reaction product. The electrophilic microbicidal activity of $^1O_2$* is similar to chlorine gas (Cl_2). However, unlike Cl_2, $^1O_2$* is a metastable electronically excited state with a microsecond half-life, and as such, reactivity is confined to within a 0.2 microns (μm) radius of its point of synthesis [22-23].

Combustive microbicidal oxygenations have exergonicities sufficient for electronic excitation. Reaction of $^1O_2$* with π bonded molecules produces electronically excited products (π*carbonyl functions) that relax to the nonbonding (n) ground state (π*→n relaxation) by emitting photons in the visible range of the electromagnetic spectrum, and as such, light, i.e., chemiluminescence, is emitted by neutrophils as an energy product of combustive microbicidal action [10-11].

2. Myeloperoxidase and contol of microbe flora

2.1 Myeloperoxidase binding selectivity and lactic acid bacteria

MPO shows selective physical binding to bacteria. Strong MPO binding was observed for all Gram-negative bacteria and some Gram-positive bacteria, especially *Staphylococcus aureus*. However, members of the lactic acid family of bacteria, especially the viridans streptococci, showed low to no binding [24]. Lactic acid bacteria (LAB) do not synthesize heme, and consequently, are cytochrome-deficient and catalase-negative. LAB lack cytochrome-dependent electron transport metabolism and produce lactic acid as a major fermentation product. Reduction of O_2 catalyzed by flavoenzymes produce H_2O_2 as a metabolic product. Many LAB are capable of accumulating millimolar (mM) concentrations of H_2O_2 in the culture medium [25-27]. The green characteristic of viridans streptococcal alpha hemolytic action is a consequence of H_2O_2 production and its action on erythrocytes in blood agar [28]. Viridans streptococci are reported to produce H_2O_2 at rate of 1-5 nmol/min/10^6 CFU [29].

Selective MPO binding correlates with selective MPO microbe killing [30]. Low or no MPO binding provides a distinct advantage to LAB in their ongoing competition with other microbes for a niche in the human flora. MPO microbicidal action results from $^1O_2$* combustive activity, and the radius of this activity is limited by the half-life of $^1O_2$*; i.e., combustive activity is limited to a radius of within 0.2 μm of the point of $^1O_2$* generation. Consequently, microbicidal combustions is focused to the sites of MPO binding and away from the H_2O_2-generating LAB [30].

2.2 MPO-LAB synergistic microbicidal action

LAB-generated H_2O_2 provides substrate for combustive killing of MPO-bound microbes. If the concentration of MPO is limited, LAB showing low or no MPO binding are spared. This relationship is the basis of MPO-LAB synergistic microbicidal action against MPO-binding microbes illustrated by MPO-*Streptococcus sanguinis* killing of *Pseudomonas aeruginosa*.

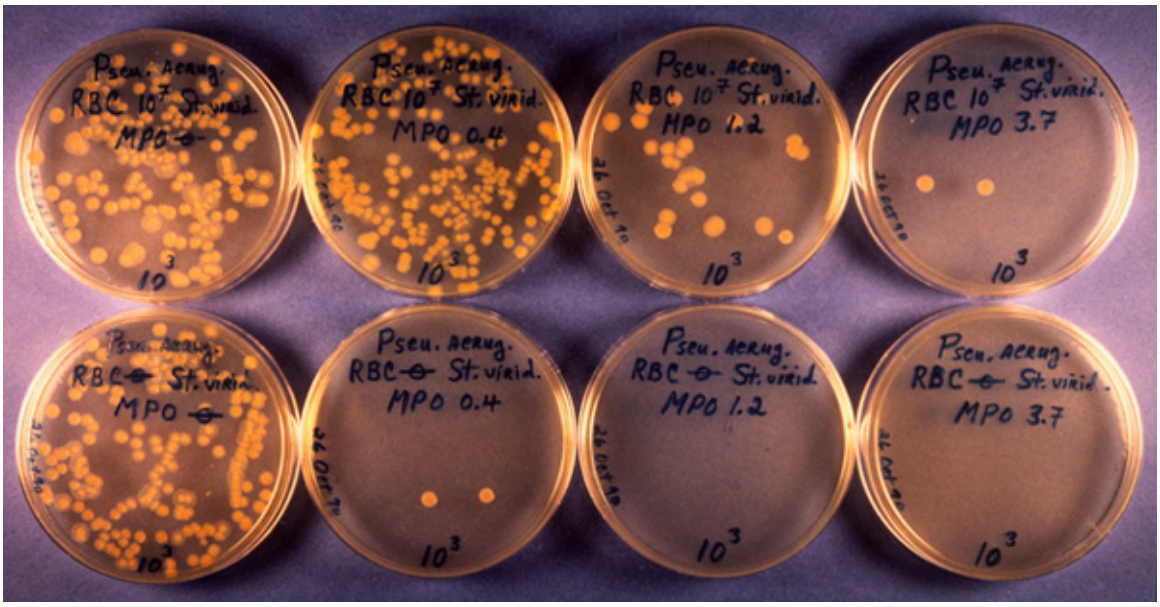

Fig. 4 Photograph of Petri plates showing *Streptococcus sanguinis*-MPO synergistic action against *Pseudomonas aeruginosa* at the 10^{-3} plate dilution for CFU determination. All testing used 10^6 *P. aeruginosa* and 10^7 *S. sanguinis* at a final volume of 1 mL. The top row of plates contained human erythrocytes (10^7/mL); the bottom of plates were without erythrocytes. From left-to-right, the concentrations of porcine MPO were: 0, 0.2, 0.6, 1.9 nM (pmol/mL). Reactive exposure was stopped at 30 min. The plates were photographed at about 36 hours [30].

In addition to its role in neutrophil anti-microbial action, MPO can serve the host in a secondary manner by providing selective advantage to beneficial LAB flora, and by controlling the population density of such flora. As shown in Figure 4, MPO-*S. sanguinis* synergistic action produced complete *P. aeruginosa* kill at an MPO concentration of 0.6 pmol/mL in the absence of erythrocytes. This and other such observations strongly support the proposition that low concentrations of MPO provide selective advantage to the viridans streptococci in their competition with MPO-binding microbes such as *P. aeruginosa* for flora dominance [30].

At relatively low H_2O_2 concentrations, inclusion of erythrocytes allows assessment of the relative specificity of MPO-dependent oxidative activity. Erythrocytes show little or no MPO binding. They are a competing substrate and

can be damaged or destroyed in oxidative reactions. The top plates in Figure 4 show the effect of erythrocytes on *S. sanguinis*-MPO synergistic killing of *P. aeruginosa*. An almost tenfold increase in MPO concentration is necessary to achieve equivalent *P. aeruginosa* killing in the presence of 10^7 erythrocytes/mL, i.e., 1.9 pmol/mL compared to 0.2 pmol/mL MPO. There was no evidence of hemolysis; no significant erythrocyte damage was observed at any concentration of MPO tested. MPO combustive oxygenations are directed specifically against the MPO-bound *P. aeruginosa*. Even at a 3 to 1 ratio of erythrocytes to *P. aeruginosa*, the efficiency of *P. aeruginosa* killing by MPO-*S. sanguinis* was only mildly decreased. Erythrocyte catalase prevents accumulation of high H_2O_2 concentrations, providing some protection to *P. aeruginosa* but proportionally higher protection to the erythrocytes and streptococci with no or low MPO-binding.

Erythrocyte inhibition of *S. sanguinis*-MPO synergistic microbicidal action is minuscule compared to their inhibition of direct H_2O_2 and HOCl microbicidal action. These chemical oxidants produce completed hemolysis and/or hemoglobin destruction at concentrations significantly lower than those necessary for direct microbicidal action; i.e., erythrocytes are more susceptible to these reactants than microbes. Selective MPO microbe binding, the short reactive lifetime of $^1O_2$*, and competitive consumption of H_2O_2 by erythrocyte catalase limits combustive oxygenation activity to the site of MPO binding and minimize bystander injury [30].

2.3 MPO availability in body fluids and spaces

Following a relatively short time within the circulating blood, neutrophils migrate into body tissues and cavities such as the mouth and vagina [2, 31-33]. These spaces are characterized by acidic pH and the presence of LAB rich flora. Neutrophils that migrate to tissue are eventually removed by macrophages, but neutrophils that migrate into the acid milieu of the mouth or vagina disintegrate, releasing their MPO content into these milieux. Treating neutrophils with mild acid is a method for solubilizing MPO from the azurophilic granules. As previously described, 10^{11} neutrophils are produced daily and their MPO content is about 4 fg MPO/neutrophil. Therefore, about 0.4 mg MPO is synthesized daily in a healthy adult [4]. MPO synthesis can increase severalfold with inflammation and following treatment with colony stimulating cytokines [6].

Saline mouth lavage of healthy human subjects consistently yields $>10^5$ neutrophils, a relatively high number considering the expectedly high rate of neutrophil clearance from the mouth. In general the oral neutrophil count is proportional to the circulating blood neutrophil count [31-32]. The vaginal space also show a similar neutrophil presence [33]. Viridans streptococci are the normal flora of the mouth; lactobacilli are the normal flora of the vaginal space. Both are LAB. In the competition for a niche within the flora, the presence of MPO favors non-MPO-binding LAB.

In effect, LAB condition the milieu to approximate the phagolysosomal space. A pH in the range of 5-6, the physiologic availability of chloride, and sufficient H_2O_2 guarantee MPO microbicidal action. This relationship is further illustrated in chronic granulomatous disease (CGD), a disorder resulting from defective neutrophil NADPH oxidase. CGD neutrophils phagocytose microbes but are unable to activate the respiratory burst metabolism required for acidification and H_2O_2 generation, and thus, are unable to kill most microbes. CGD patients suffer from severe and recurrent staphylococcal, coliform and fungal infections, but do not show increased susceptibility to streptococcal infections [34]. Whereas killing and chemiluminescence are not observed following phagocytosis of staphylococci, coliforms and fungi, phagocytosis of viable streptococci by CGD neutrophils results in microbicidal action and chemiluminescence. CGD neutrophils carry out phagocytosis and azurophilic granule fusion producing a MPO-rich phagolysosomal space. The metabolism of the phagocytosed streptococci substituted for defective NADPH oxidase; i.e., streptococci produced the acidity and H_2O_2 required to drive MPO production of HOCl and $^1O_2$* resulting in combustive microbicidal action and chemiluminescence [34]. Within the MPO-rich phagolysosomal space there is no necessity for selective binding. In body spaces, such as the mouth and vagina, specificity of MPO binding and killing favours dominance of LAB in flora composition.

3. Oxidase-MPO formulations for prevention and control of infection

The schematic of Figure 2 depicting the phagolysosomal microbicidal apparatus of the neutrophil can be readily modified to describe the microbicidal action of neutrophil-free MPO in body fluid and spaces. LAB metabolism substitutes for NADPH oxidase by providing the acid milieu and generating the H_2O_2 substrate for MPO microbicidal action. When MPO concentration is limited, binding selectivity insures that benign LAB are spared and competing pathogens are eliminated.

3.1 Approximating the phagolysosomal MPO microbicidal system

The essential features of phagocyte and extra-phagocyte MPO-mediated microbicidal action are the same: (1) MPO, (2) a source of H_2O_2, (3) an acidic milieu, and (4) sufficient chloride. Potent MPO microbicidal action is obtained by simply applying MPO and providing H_2O_2 in an acidic saline solution. However, H_2O_2 in high concentration should be

avoided as it can inhibit peroxidase activity. Chloride, specifically the ratio of chloride to H_2O_2, protects MPO from the inhibitory effect of H_2O_2.

The phagolysosomal system is best approximated when an oxidase is used to generate H_2O_2 at a controllable rate. Rate control over the quantity of H_2O_2 produced can be achieved by adjusting the quantity of oxidase and/or the concentration of substrate required by the oxidase. A relatively large quantity of H_2O_2 can be generated over time, but the rate of generation can be adjusted to avoid high peak H_2O_2 concentrations that could inhibit MPO action. There are many different substrate-specific oxidases applicable to H_2O_2 production. Ideally the oxidase should be robust with a substrate requirement that doesn't interfere with MPO combustive oxygenation activity.

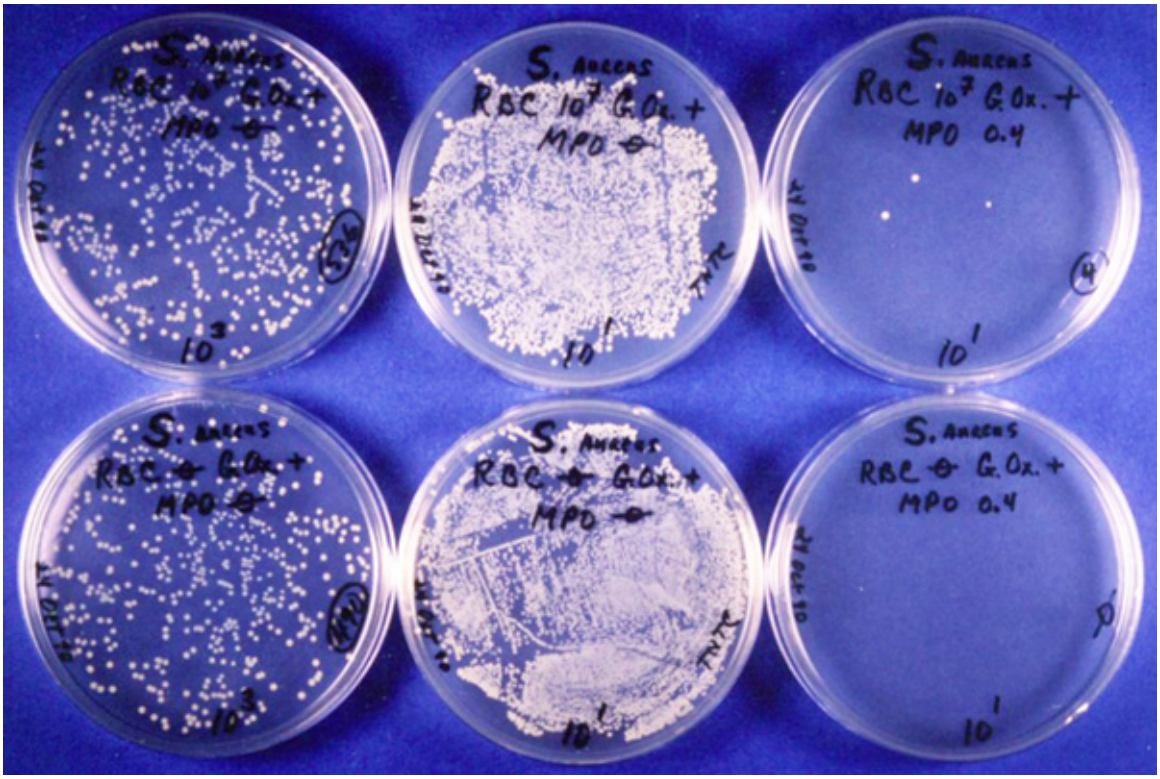

Fig. 5 Photograph of Petri plates showing GO-driven MPO killing of *Staphylococcus aureus*. The top row of plates shows killing in the presence human erythrocytes (10^7/mL); the bottom row of plates were without erythrocytes. The plates on the left are shown at the 10^3 dilution in the absence of MPO (the plate used CFU counting). The plates in the middle show activity at the 10^1 dilution where the colonies were too numerous to count in the absence of MPO and are included for comparison to activity with MPO. The plates on the right show the effects of 0.2 nM (pmol/mL) porcine MPO on *S. aureus* CFU at the 10^1 dilution. All plates contained glucose oxidase (GO) from *Aspergillus niger* in excess (100 nM) with a limiting concentration, 5.6 mM (1 mg/mL), of glucose as substrate. The final volume of the suspension was 1 mL; reactive exposure was stopped at 30 min [30].

Glucose oxidase (GO) has a long history of use in diagnostic applications and is an obvious candidate oxidase to drive MPO microbicidal action. As illustrated in Figure 5, GO-driven MPO exerts a potent microbicidal action against *S. aureus*. In the absence of MPO but with GO plus glucose as the H_2O_2 generator, the *S. aureus* count was $4.9x10^6$ CFU/mL in the absence of erythrocytes and $5.4x10^6$ CFU/mL in the presence of 10^7 erythrocytes. *S. aureus* did not show significant susceptibility to GO-glucose in the absence or presence of erythrocytes. When the GO-glucose system included 0.2 pmol/mL MPO, complete *S. aureus* killing was realized in the absence of erythrocytes and >99.9% killing was realized in the presence of erythrocytes. No erythrocyte hemolysis was observed under the conditions of testing. As such, MPO oxygenation activity was focused to the target *S. aureus* minimizing erythrocyte damage.

3.2 GO-MPO microbicidal formulation for prevention of surgical site and wound infections

An antimicrobial system, i.e., E-101, comprised of GO purified from *Aspergillus niger* and purified porcine MPO, has been formulated and is under testing as a solution for prevention of surgical site and wound infections. The formulation is mildly acidic, pH 6.5, and contains sufficient chloride to support MPO action. On contact with glucose, GO produces the H_2O_2 necessary to drive MPO microbicidal action. E-101 has been shown to exert potent microbicidal action against a broad spectrum of microbes including strains showing antibiotic resistance [35-36].

E-101 has been shown to be safe and effective in rat and pig models of skin infection [37-38]. In addition, E-101 was non-irritating in conventional cumulative human Phase 1 skin irritation testing [39]. Additional applied research is ongoing.

Acknowledgements The support of Exoxemis, Inc is gratefully acknowledged.

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