Determination of lactoperoxidase system components in adult and neonatal human saliva

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ABSTRACT

The lactoperoxidase system plays a significant role in innate immunity by regulating the commensal microbiome while inhibiting pathogens, especially in breastmilk and saliva. Samples were collected from neonates (n=15) using sterile swabs and directly from adults (n=19). Salivary lactoperoxidase and thiocyanate concentrations were determined in adult humans and neonates. Samples were analyzed for lactoperoxidase activity using a sensitive enzymatic/colorimetric assay. The activity of peroxidase (p=0.001) and thiocyanate (p=0.004) were significantly higher in adults than in neonates. There were some variability between the obtained data, but this variability might be due to the sample stimulus and nutritional status. In conclusion, the samples collected for this study exhibited efficient peroxidase activity and sufficient thiocyanate concentration, which may have antibacterial activity. The hydrogen peroxide for this system can be provided by oral commensal bacteria or by breast milk oxidases.

Keywords: Peroxidase, hydrogen peroxide, thiocyanate, innate immunity.

INTRODUCTION

Saliva contains a variety of specialized secretory proteins and enzymes. The immunologic proteins of saliva are IgA, IgM and IgG, with secretory IgA representing the highest concentration (de Almeida et al., 2008). The non-immunologic antibacterial components of saliva include several enzymes, in particular lactoperoxidase (which plays a significant role in the generation of oxygen radicals), lysozyme and lactoferrin (Aps et al., 2005; Dodds et al., 2005). Salivary enzymes also include other enzymes such as amylase, lipase, protease, DNase and RNase (de Almeida et al., 2008).

Several body fluids such as tears, milk and saliva have lactoperoxidase activity. Lactoperoxidase is an enzyme that plays a role in an innate immunity defense mechanism, particularly in saliva and milk (Reiter et al., 1981; Silankove et al., 2005). At a physiological concentration, peroxide free radicals can combine with thiocyanate (SCN-), which is abundant in adult and infant saliva, through catalysis by the enzyme lactoperoxidase (LPO) and also present in milk and saliva to produce a more potent anti-bacterial free radical hypothiocyanite (OSCN-): this is known as the 'lactoperoxidase system' (Wijkstrom-Frei et al., 2003; Welk et al., 2011). This complex system may provide the infant mouth, gastrointestinal tract and upper respiratory system with continuous passive protection against invading bacteria.

Reactive oxygen species (ROS) are also a well-known antimicrobial agent (Juven et al., 1996). Interestingly, when human cells are exposed to pathogens, phagocytic white blood cells present in localized inflammation within human tissues produce various concentrations of ROS as a part of the first-line defense against microbial invasion (Alfadda et al., 2012). In addition, a similar system is also present in natural products. The LPO system was recognized as critical in the dairy industry for the preservation of raw milk (Haddadin et al., 1996). Furthermore, honey contains glucose oxidase, an enzyme secreted by the pharyngeal gland of bees; glucose oxidase
reacts with glucose, subsequently generating the antibacterial hydrogen peroxide (Al-Shehri, 2017).

Bacteria colonization in neonates starts immediately after birth (Cephas et al., 2011). Several factors control this colonization, which subsequently prevents pathogenic infections. The LPO system has been proven to be one of these factors and it is functional in saliva and breastmilk (Al-Shehri et al., 2015). Therefore, breast-feeding during early infancy is crucial for infants.

This current study is a part of other studies investigating the role of ROS in innate immunity, particularly, in neonates and infants. The aim of this study is to determine and evaluate the levels of thiocyanate and peroxidase activity in neonatal saliva and compare them to the levels in adults.

**MATERIALS AND METHODS**

**Human saliva samples**

The study protocols received prior written approval from the Human Research Ethics Committees (HREC) of Mater Health Services (MHS) with informed consent (Number 2012_01LNR).

Saliva collection from infants (n=15) and adults (n=19) was considered to have 'minimal' risk of harm – in fact, we found that none of the infants showed any distress during collection of saliva using soft cotton swabs, which required approximately 30 s. Analyses of the saliva samples as well as, the validation of analysis and limit of detection of salivary nucleotide metabolites using HPLC-tandem mass spectrometry (LC-MS/MS) were conducted as previously described (Al-Shehri et al., 2013). Samples were collected from vaginally delivered, full-term neonates identified as Caucasian.

**Peroxide and calibration curve preparation**

Hydrogen peroxide (Merck Pty Ltd), 30% v/v, 9 M, was used as a calibration standard by dilution to approximately 9 mM in 100 mM phosphate-buffer saline (PBS) using a quartz cuvette. The concentrations of hydrogen peroxide were accurately determined spectrophotometrically in duplicate at 240 nm assuming a molar absorptivity (ε) of 43.6 M⁻¹cm⁻¹.

The assay reagent contained 100 μM of Ampliflu Red (Sigma-Aldrich Pty) and 200 μM hydrogen peroxide in PBS. Buffers and aqueous reagents for all experiments were prepared from MilliQ distilled water. In the presence of peroxidase, hydrogen peroxide was reacted with Ampliflu Red (10-acetyl-3,7-dihydroxyphenoxazine) to produce the red-fluorescent oxidation product resorufin. This product was measured fluorometrically at 544 nm excitation and 590 nm emission.

**Lactoperoxidase assay**

For the assay of peroxidase activity in saliva, 50 μl saliva diluted 1:15 in PBS was added into a 96-microplate. The reaction was started by adding 50 μl of 100 μM Ampliflu Red solution containing 200 μM of hydrogen peroxide at a pH of 7.5. All samples and linked assays were incubated in flat-bottom microtiter plates (Becton Dickinson) at 37°C with shaking in a temperature-controlled FLUOstar Omega fluorimeter (BMG Labtech) for 60 min. For each sample, a corresponding assay substrate blank (excluding human sample) was subtracted from each data point.

**Salivary thiocyanate assay**

The thiocyanate assay was based on a spectrophotometric method developed for human saliva using the ferric nitrate method adapted for microtiter plates (Hovinen et al., 1999).

**Statistical analysis**

The linear regression curve method was used to generate the study's standard curve and the appropriate equation. Mean values were compared between the two different groups using an unpaired, two-tailed t-test. A p-value of 0.05 was the cut-off for statistical significance. All analyses were performed using GraphPad Prism 7 software.

**RESULTS**

Table 1 shows information on the study participants. The activity of lactoperoxidase was calculated from a calibration curve showing leaner correlation (y=67.49x, r²=.937). The lactoperoxidase activity in neonatal saliva during the first few hours showed a narrow range (mean ± SD, 2.2±4.8) with the exception of two participants showing higher levels (Figure 1). The enzyme activity in the adult samples was higher than in neonates (20.9±16.4). This variation between the two groups was statistically significant (p=0.001).

The concentration of thiocyanate in the saliva of neonates was found to be 0.83±0.691 mM (mean ± SD). This concentration was significantly lower (p=0.004) than the level found in adult saliva, 2.24±1.62 (Figure 2).

**DISCUSSION**

The innate immune system is the first-line defense against invading pathogens and it is particularly important in preventing bacterial and viral infections (Marodi, 2006). LOP is an essential innate immunity system for its ability to generate ROS and it is a well-characterized component.
Table 1: Physical information on the participating neonates.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
<th>P-value</th>
</tr>
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<td>N</td>
<td>4</td>
<td>8</td>
<td>12</td>
<td>n.s.</td>
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<tr>
<td>Gestational age (weeks)</td>
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<td>Range</td>
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<td>39-42</td>
<td>37-42</td>
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<tr>
<td>Birth weight (g)</td>
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<tr>
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<td>3785±3354</td>
<td>3690±332</td>
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<tr>
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<td>3150-4240</td>
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</tr>
<tr>
<td>Age (hours)</td>
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</tr>
<tr>
<td>Mean ± SD</td>
<td>35±13</td>
<td>34±16</td>
<td>36±12</td>
<td>0.9 (n.s.)</td>
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<tr>
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<td>26-60</td>
<td>15-60</td>
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</table>

Figure 1: Lactoperoxidase activity in adult and neonatal human saliva.

of mammary and salivary gland secretions (Welk et al., 2009).

In this study, we showed that peroxidase is enzymatically active in human saliva and adults have significant enzyme activity as compared with neonates. The data demonstrated varying but sometimes very high peroxidase activity. Interestingly, the neonates with higher salivary peroxidase activity were those with a younger age and shorter gestational age among the neonates studied. The thiocyanate concentration was found to be in the millimol arrangement where adult thiocyanate levels were significantly higher than that of neonates. This variation between the results might be due to factors such as the flow rate, type of stimulus and nutritional status of the participant.

While thiocyanate and hydrogen peroxide are the substrates for peroxidase, hydrogen peroxide is sourced to the LPO system by oral commensal bacteria. Some Streptococcus species found in oral surfaces can generate physiological concentrations of peroxide (Tenovuo et al., 1986; Hyyppa et al., 1989).

Interestingly, another recent study showed that neonatal
Figure 2: Salivary thiocyanate concentration (in millimolars).

saliva contains high levels of the metabolites xanthine and hypoxanthine (Al-Shehri et al., 2013). They are substrates of the enzyme xanthine oxidase, which is highly abundant in breast milk. During breast-feeding, the mixing of neonatal saliva with breast milk generates micromolar levels of hydrogen peroxide, a necessary substrate for the activation of the 'lactoperoxidase system' to produce additional bactericidal ROS (Al-Shehri et al., 2015). These metabolites enable unique antibacterial activity within the neonatal mouth at a time when other immune mechanisms are not fully developed.

In summary, human neonatal and adult salivary gland secretions contain the components of the LPO system, which has previously been proven to be effective against a variety of respiratory pathogens and thus, may offer new approaches to boosting innate immunity, especially in neonates during a critical period of human life.

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REFERENCES


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