The Effect of Dilution on the Rate of Hydrogen Peroxide Production in Honey and Its Implications for Wound Healing

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ABSTRACT

Objective: Honey is an effective antiseptic wound dressing, mainly the result of the antibacterial activity of hydrogen peroxide that is produced in honey by the enzyme glucose oxidase. Because the rate of production of hydrogen peroxide is known to vary disproportionately when honey is diluted, and dilution of honey dressings will vary according to the amount of wound exudate, it is important to know more about the production of hydrogen peroxide at different concentrations of honey.

Design: The rates of hydrogen peroxide production by honey with respect to honey dilution were measured in eight different samples of honey from six different floral sources.

Settings: Honey Research Unit, Waikato University, Hamilton, New Zealand.

Main Results: The maximum levels of accumulated hydrogen peroxide occurred in honey solutions diluted to concentrations between 30% and 50% (v/v) with at least 50% of the maximum levels occurring at 15–67% (v/v). This is equivalent to a 10 cm $\times$ 10 cm dressing containing 20 mL of honey becoming diluted with 10 to 113 mL of wound exudate. Maximum levels of hydrogen peroxide reached in the diluted honeys were in the range of 1–2 mmol/L.

Conclusion: Significant antibacterial activity can be maintained easily when using honey as a wound dressing, even on a heavily exuding wound. Concentrations of hydrogen peroxide generated are very low in comparison to those typically applied to a wound, thus, cytotoxic damage by hydrogen peroxide is very low.

INTRODUCTION

HONEY HAS LONG BEEN DOCUMENTED as having healing properties (Majno, 1975; Ransome, 1937) and more recent research has shown that honey can be effective for clearing infections in a wide range of wounds, including abscesses, surgical wounds, traumatic wounds, burns, and ulcers of varied etiology. Many of these often do not respond to conventional therapy (Molan, 1998; 2001). Minimum inhibitory concentrations of 3%–4% (v/v) honey for 58 isolates of *Staphylococcus aureus* from infected wounds (Cooper et al., 1999) and of 5.8%–9% (v/v) honey for 20 isolates of *Pseudomonas* spp. from infected wounds (Cooper and Molan, 1999a), found in testing with honey selected to have a near-median level of antibacterial activity, reveal that honey has potential for use as an alternative to the antibiotics currently being used

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for clearing infections in wounds. Such interest in the use of honey as a wound dressing is strengthened by the widespread development of antibiotic-resistance in bacteria (Greenwood, 1995) and the knowledge that honey is fully effective against such antibiotic-resistant bacteria (Molan and Brett, 1994).

With the exception of *manuka* honey, the antibacterial activity of most honeys seems to be primarily the result of hydrogen peroxide that is produced by the action of glucose oxidase in honey (Adcock, 1962; White et al., 1963). Hydrogen peroxide is an effective antimicrobial agent if present at a sufficiently high concentration (Roth et al., 1986), but at higher concentrations causes cellular and protein damage in tissues by giving rise to oxygen radicals (Cochrane, 1991; Simon et al., 1981). The acceleration of the healing process observed when honey is used as a dressing on wounds (Molan, 1998, 2001) may also be the result of the hydrogen peroxide produced in honey. There is a large amount of evidence for hydrogen peroxide being involved in many cell types in the body as a stimulus for cell multiplication, with hydrogen peroxide being produced as part of the normal inflammatory response to injury or infection, and serving to stimulate the growth of fibroblasts and epithelial cells to repair the damage (Burdon, 1995). Hydrogen peroxide also stimulates the development of new capillaries in wound tissue (Tur et al., 1995). It has been proposed that low concentrations of hydrogen peroxide might be used to stimulate wound healing in place of the recombinant cell-growth factors currently being used (Burdon, 1995); however, it has been pointed out that this is feasible only if the concentration of hydrogen peroxide could be carefully controlled to avoid tissue damage (Chung et al., 1993).

There are various reports that indicate that rates of hydrogen peroxide production in honey can vary greatly (White et al., 1962, 1963; White and Subers, 1963), it increases disproportionately with different degrees of honey dilution (White et al., 1962). In addition, different wounds have different amounts of exudates. Thus, honey applied to wounds is diluted to different degrees, resulting in varied levels of hydrogen-peroxide production. It is therefore crucial to know more about the rate of hydrogen peroxide produc-

tion as honey is diluted so that an appropriate level of hydrogen peroxide can be achieved on a wound.

MATERIALS AND METHODS

Preparation of honey solutions

The honeys used in this study were selected to represent a range of different floral sources. All had been shown to have medium to high antibacterial activity that results from hydrogen peroxide. The eight honeys used were: two pasture honeys (mixed sources), one clover (*Trifolium repens*) honey, one bush honey (mixed source), one *rewarewa* (*Knightia excelsa*) honey, and three composite honeys that were made by pooling honeys of a single type [*rewarewa*, wildflora or ling heather (*Calluna vulgaris*)] from many (20–30) different batches. These honeys were assumed to be representative of honeys from their respective floral sources. The honeys were centrifugally extracted, well-mixed, and stored undiluted at 4°C in the dark.

Honey solutions were prepared immediately prior to testing by diluting honey to the required concentrations (10, 20, 30, 40, 50, 60, or 70%, v/v) using 3-(N-morpholine)propanesulfonic acid (MOPS)-acetate buffer (0.1 mol/L, pH 6.0) at 37°C. Each solution was adjusted to a pH of 6.0 with 0.01mol/L NaOH as necessary. All samples were then incubated for 30 minutes at 37°C in a shaking water bath that allowed aeration of the solutions. Incubation was carried out in the dark because both hydrogen peroxide and glucose oxidase are light-sensitive (White and Subers, 1964a).

Samples of *rewarewa* and ling heather honey were also diluted to 50% in MOPS-acetate buffer, adjusted to pH 6.0 and incubated at 37°C for up to 72 hours. Levels of accumulated hydrogen peroxide were measured after 2, 4, 8, 24, 32, 48, and 72 hours to determine the levels reached after prolonged incubation.

Measurement of hydrogen peroxide production

A YSI model 53 oxygen monitor with a recorder attached (Sekonic SS-25OF, Sekonic, Japan) was used to measure the amount of hydrogen peroxide accumulated in the honey so-

lutions over the 30-min incubation period. This was measured as the amount of oxygen evolved when catalase was added to decompose the hydrogen peroxide. Water heated to 37°C was circulated around the sample chamber. All honey solutions were diluted to a concentration of 5% (v/v) prior to assay. This ensured that all solutions within a honey series showed negligible oxygen consumption due to glucose oxidase activity during the 2- to 3-minute assay period. This was confirmed by measuring uptake of oxygen by 5% honey solutions, all honeys showing negligible oxygen consumption (<0.001 mmol/min).

For the assay, a 3-mL volume of 5% honey solution was added to the sample chamber of the oxygen monitor and stirred after insertion of the electrode plunger. Once the oxygen meter displayed a steady reading (indicating temperature equilibrium), 0.1 mL of catalase (Sigma, St. Louis, MO; catalogue number C-9322; 14.34 Units/mL) was injected into the solution down the side of the plunger. The continuous stirring ensured rapid mixing of the catalase with the other assay constituents. The total increase in dissolved oxygen was then measured and recorded. Each concentration of honey was incubated in duplicate and assayed in triplicate. The catalase solutions used were made daily and protected from light.

The increase in dissolved oxygen was related to the concentration of hydrogen peroxide accumulated in the honey by comparison with a standard curve presenting standards of known concentrations of hydrogen peroxide against the oxygen evolved when the standard solutions were assayed as above (the standard solution replacing the honey). Standard concentrations of 0.1–0.5 mmol were used. Solutions were prepared fresh daily by dissolving a stock solution of hydrogen peroxide in MOPS-acetate buffer (0.1 mol/L, pH 6.0 at 37°C) made with MilliQ deionized water (MilliQ Ultrapure Water Systems, Millipore). All solutions were protected from light. The precise concentration of the standard stock solution was determined by titration against potassium iodide. To check that honey did not interfere with the oxygen electrode, the standard curve was also produced using hydrogen peroxide made up in a 5%-honey solution diluted immediately prior to assay.

To check that glucose oxidase activity of the honeys was maintained at a constant level over the 30-minute incubation period, the experiment was repeated with a 15-minute incubation period.

To check that the pH of the honey solutions did not change significantly during the 30-minute incubation period due to production of gluconic acid (formed in the reaction of glucose oxidase acting on glucose and oxygen), the pH was measured at the end of the incubation.

RESULTS

The standard curves produced for hydrogen peroxide in the buffer and honey–buffer solutions were identical. The relationship between the increase in dissolved oxygen and hydrogen peroxide concentration was linear ($R^2 = 0.993$) in the total range measured (0.1 to 0.5 mmol hydrogen peroxide).

The levels of hydrogen peroxide accumulated in the 15-min incubation period were exactly half ($\pm 4\%$) of those accumulated in the 30-min incubation period. The 30-min incubation time chosen for this study was therefore satisfactory, with no measurable drop in glucose oxidase activity occurring during this time.

A comparison of the levels of hydrogen peroxide accumulated by each of the honey samples (Fig. 1) shows that maximum accumulation occurred at honey concentrations of 30%–40% honey for all honeys except two, which showed maximum levels at 40%–50%. At least 50% of the maximum levels of hydrogen peroxide production were still reached in a broad range of honey concentrations: as an average for the 8 honeys tested, this range was 15%–67% (v/v).

Prolonged incubation of the composite rewarewa and composite ling heather honeys at a concentration of 50% revealed that hydrogen peroxide concentrations initially increased before decreasing to 0 (Fig. 2). Large differences between the two honeys were observed, however. The composite *rewarewa* sample reached a maximum level of accumulated hydrogen peroxide (3.65 mmol) after 24 hours of incubation. By contrast the ling heather reached its maximum level of accumulation after only 4 hours with total loss of accumulated hydrogen peroxide after 24 hours.
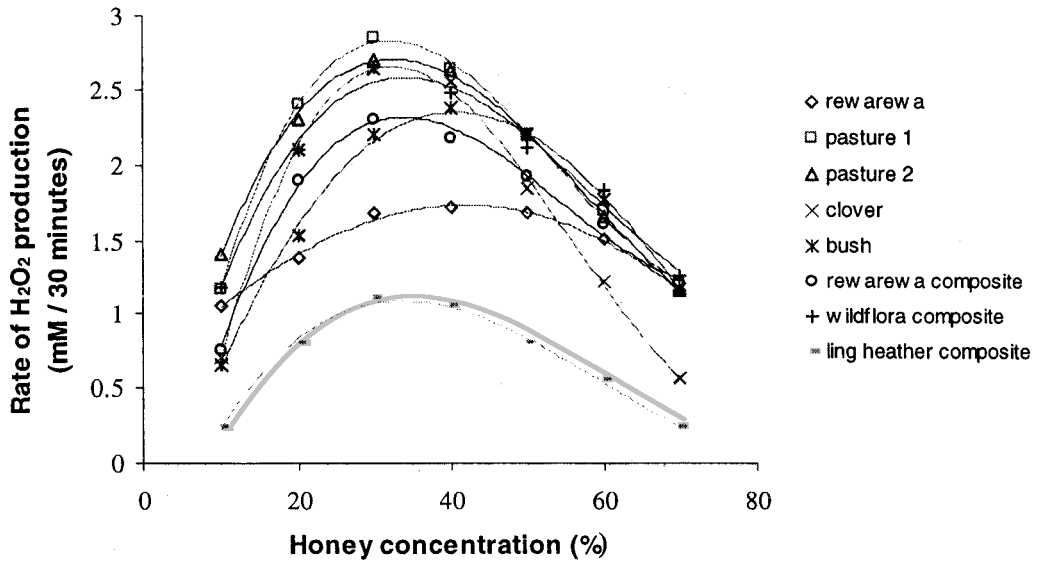
FIG. 1. Hydrogen peroxide production by honey at various degrees of dilution [mean $\pm$ standard deviation, $n = 3$), expressed as mmoles hydrogen peroxide per liter of honey solution per 30 minutes. The measured increases in dissolved oxygen on addition of catalase were converted to the corresponding amounts of hydrogen peroxide by reference to a calibration curve produced with hydrogen peroxide standards.

DISCUSSION

The results presented in this study provide valuable information for the use of honey as a wound dressing and provide insights into the changes in activity that may occur upon dilution of honey by wound exudates.

The literature has long suggested that the rate of hydrogen peroxide production by glucose oxidase in honey depends largely on the degree of honey dilution (White et al., 1963) and that little or no production of hydrogen peroxide occurs in full strength honey (Radwan et al., 1984; White et al., 1963). The current
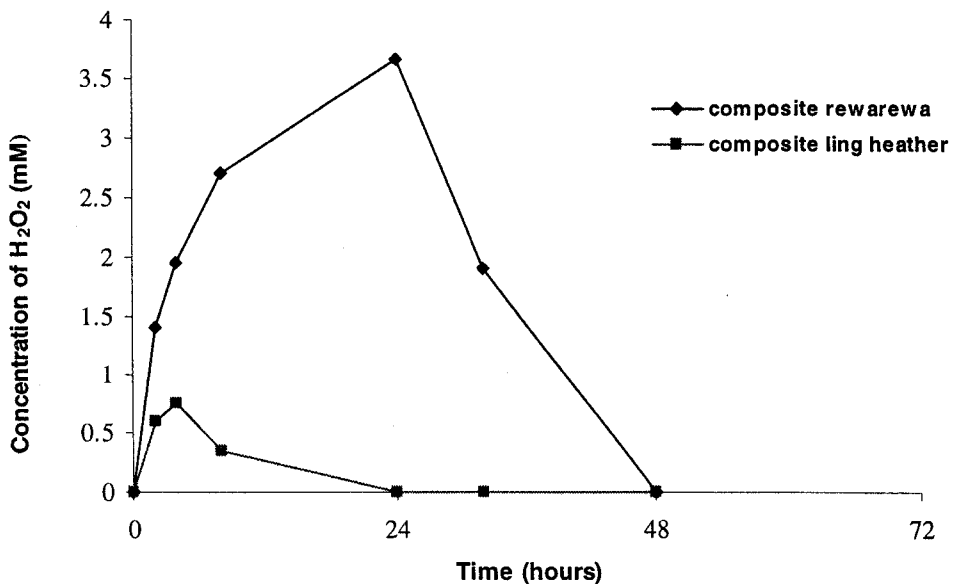

FIG. 2. Accumulation of hydrogen peroxide (mmol) with respect to time in a 50% composite *rewarewa* honey sample and a 50% composite ling heather sample incubated at 37°C for 72 hours. Hydrogen peroxide was measured using the oxygen electrode, as described in the text.

work, using eight honeys from different floral sources, confirms this theory and shows that maximum accumulation of hydrogen peroxide occurs at honey concentrations of 30%–50%, despite the concentration of enzyme and substrate being decreased as the honey is diluted from full strength honey. The glucose concentration is suboptimal for the enzyme in honey solutions at concentrations below 75% v/v (Molan, 1992).

The reason for low glucose oxidase activity in higher concentrations of honey is unknown, although previous research has indicated that it is not a result of glucose oxidase inhibition by constituents of honey such as fructose, glucose, sucrose, maltose, benzoate, gluconate, gluconolactone, or a range of minerals (Schepartz and Subers, 1964). It has been suggested that it may be the result of the low pH of full-strength honey being outside the range needed for activity of the glucose oxidase (White and Subers, 1963), but this can be ruled out because the incubation of honeys in the present study all occurred at pH 6, the optimum pH for glucose oxidase in honey (Schepartz and Subers, 1964).

Although there were differences between the eight honeys tested, all honeys accumulated significant levels of hydrogen peroxide (greater than 50% of the maximum level accumulated by each honey) over a wide range of honey concentrations. This range of honey concentrations, 15%–67%, on average, is equivalent to a 10 cm × 10 cm dressing containing 20 mL of honey becoming diluted with 10 to 113 mL of wound exudate. Because large volumes of exudates cannot be physically contained in dressings on exuding wounds, dressings would have to be changed before honey became diluted to a concentration below that which gives the maximum rate of hydrogen-peroxide production.

Honey selected for its hydrogen peroxide activity and used as an antiseptic dressing on nonexuding wounds would need to be diluted a little prior to use, although some dilution would possibly occur due to the osmotic withdrawal of wound fluid by honey. An assessment of the amount of fluid likely to come from the wound would allow it to be concluded whether or not dilution of honey prior to application is required.

It would also be advantageous if honeys chosen for use in antiseptic dressings were sterilized prior to use to prevent any microbial contamination. Molan and Allen (1996) have shown gamma irradiation to be an effective method of sterilization as there is no resultant loss of antibacterial activity. By contrast, heat treatments rapidly destroy all antibacterial activity due to inactivation of the glucose oxidase enzyme (White and Subers, 1964b). Gamma-irradiated honeys are commercially available from numerous suppliers.

The levels of hydrogen peroxide that were measured in this study are consistent with results obtained for different honeys in other studies. Levels of 0 mmol–2.12 mmol were recorded for 90 samples diluted to 14% and incubated for 1 hour (Roth et al., 1986). A similar study of 31 samples found values ranging from 0 mmol–0.95 mmol (Bogdanov, 1984). Such variation among honeys seems to be strongly related to the level of catalase activity present in the honey (Dustmann, 1971; Schepartz and Subers, 1966). However, antibacterial testing in our laboratory has shown that such concentrations of hydrogen peroxide are effective for killing bacteria. Indeed, the *rewarewa* honey used in this study was highly active, with minimum inhibitory concentrations of only 2.6%–7.1% (v/v) for the seven common wound-infecting species of bacteria tested (Willix et al., 1992). Thus, only low concentrations of active honey need be applied to a wound.

The use of hydrogen peroxide as a means of clearing infections in wounds has often been discouraged as a result of the cytotoxicity that can occur at millimolar concentrations (Lineweaver et al., 1985; Thomas, 1990). By contrast, honey has been shown to be an effective antimicrobial agent by supplying low levels of hydrogen peroxide to wounds continuously over time rather than as a large amount at the time of dressing. Whereas the amount of hydrogen peroxide accumulated over a 30-minute period in the laboratory was in some honeys in excess of 2.5 mmol (and on prolonged incubation could reach levels approximately twice as high), the amount that would be present on a wound would depend on the balance between the rate of its production and the rate of its destruction.

Indeed, as this study has indicated, hydrogen peroxide levels do not continue to increase over time, but rather increase to a point before decreasing to eventually give no measurable hydrogen peroxide. Preliminary results indicate that this is, in part, the result of decreasing glucose oxidase activity over time as the rate of oxygen uptake by the enzyme decreases over time (unpublished data). Reasons for this inhibition of glucose oxidase activity are unknown, however, it is likely to be related to the fact that lower rates of hydrogen peroxide are observed in higher honey concentrations. Whereas the information for accumulated hydrogen peroxide given in Fig. 2 is valuable, much more work needs to be undertaken before general conclusions can be drawn. Honeys from other floral sources with different rates of hydrogen peroxide production need to be assessed and the effect of other plasma components need to be determined.

Plasma enzymes such as catalase and glutathione peroxidase are likely to decrease the levels of hydrogen peroxide reached on wounds significantly (Cooper and Molan, 1999b; Postmes et al., 1993), although recent work in our laboratory indicates that the hydrogen peroxide is not completely inactivated by plasma (manuscript in preparation). In addition, changes in pH that result from the dilution of honey with varying degrees of plasma are likely to affect the rate of hydrogen-peroxide production by glucose oxidase. The degree to which the honey is diluted is also crucial to the overall survival time of the hydrogen peroxide produced and, thus, the levels reached. The data given in Fig. 2 are a result of dilution of honey to a concentration of 50%, not the concentration that produced maximal production. Prolonged incubation of honey at 30%–40% (the range for maximal hydrogen peroxide production) should generate higher concentrations of hydrogen peroxide, therefore leading to a prolonged interval before total loss of hydrogen peroxide is observed. However, the addition of honey to a wound is not going to result in one dilution, but rather in varying dilutions over time as the amount of exudate in it increases. Careful evaluation of the honey to be used on a wound and the times required between dressing changes must be undertaken to allow full effectiveness of hydrogen peroxide-induced wound healing properties.

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