The Effect of Gamma-irradiation on the Antibacterial Activity of Honey

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Abstract

There is increasing usage of honey as a dressing on infected wounds, burns and ulcers, but there is some concern that there may be a risk of wound botulism from the clostridial spores sometimes found in honey. It is well-established that the antibacterial activity is heat-labile so would be destroyed if honey were sterilized by autoclaving, but the effect of gamma-irradiation on the antibacterial activity of honey is not known. Therefore an investigation was carried out to assess the effect on the antibacterial activity of honey when the honey was subjected to a commercial sterilization procedure using gamma-irradiation (25 kGy).

Two honeys with antibacterial activity due to enzymically-generated hydrogen peroxide and three manuka honeys with non-peroxide antibacterial activity were investigated. The honeys were tested against *Staphylococcus aureus* in an agar well diffusion assay. There was no significant change found in either type of antibacterial activity resulting from this form of sterilization of honey, even when the radiation was doubled (to 50 kGy).

Testing of honey seeded with spores of *Clostridium perfringens* and *C. tetani* (10 000 and 1000 spores g^{-1} of honey, respectively) showed that 25 kGy of gamma-irradiation was sufficient to achieve sterility.

The use of honey as a topical antibacterial agent has been re-discovered by the medical profession in recent times (Zumla & Lulat 1989) and is gaining acceptance for the treatment of surface infections such as ulcers and bed sores (Hutton 1966; Blomfield 1973; Bloomfield 1976; Keast-Butler 1980), and those resulting from burns, injuries and surgical wounds (Bulman 1955; Cavanagh et al 1970; Lawrence 1976; Armon 1980; Braniki 1981; Dumronglert 1983; Effem 1988; Green 1988; McInerney 1990). A feature of the usage of honey as a dressing that is noted in many of these reports cited is the rapid clearance of infection, in many cases from wounds that had not responded to various forms of conventional treatment.

There have been numerous investigations of the antibacterial activity of honey reported, presenting a large amount of evidence that this activity is over and above that due to the acidity and the high osmolarity of honey (reviewed by Molan 1992 a,b). Although there is wide variability between the antibacterial activies of different honeys, some are very potent antibacterial agents (Allen et al 1991). Most honeys owe their antibacterial activies primarily to hydrogen peroxide produced enzymically in-situ by the glucose oxidase present (Molan 1992a), but one type of honey, from manuka (*Leptospermum scoparium* J. R. et G. Forst., family: *Myrtaceae*), has been found to have a high level of non-peroxide antibacterial activity (Allen et al 1991). Willix et al (1992) tested both the non-peroxide antibacterial activity of a manuka honey and the hydrogen peroxide activity of another honey against the major wound-infecting species of bacteria: complete inhibition of growth of all seven species tested was achieved with concentrations below 11% (v/v) of these honeys which had average levels of antibacterial activity. Such concentration could be

expected to be easily maintained on a wound surface even with suppurating wounds.

The use of honey as a wound dressing has been argued against, however, on the grounds that the risk of it possibly causing wound botulism is unacceptable (Mossel 1980). Clostridia are widely distributed in nature so their presence in traumatic wounds is to be expected, yet there is a very low incidence of wound botulism, which raises questions about the pathogenesis of the disease (Merson & Dowell 1973). However, there are several reports of honey sometimes containing spores of *Clostridium botulinum* (Sugiyama et al 1978; Midura et al 1979; Huhtanen et al 1981; Kautter et al 1982; Nakano & Sakaguchi 1991), so there is a definite risk of introducing the microorganism into wounds if honey is used as a dressing.

Mossel (1980) has suggested that the risk be avoided by having hospital pharmacists prepare a sterile hydrolysate of sucrose with the same water content as honey, but this implies that it is only the osmolarity that is of any importance in the antibacterial activity. The study of Willix et al (1992) has shown clearly that other antibacterial factors in honey are far more important. Therefore it is worth considering whether honey can be sterilized for use as a wound dressing. The glucose oxidase activity which generates the hydrogen peroxide is very labile to heating (Molan 1992b). Although the non-peroxide activity of manuka honey is relatively stable to heating (Molan & Russell 1988), there is some loss on autoclaving (unpublished findings), and there would also be complete loss of any hydrogen peroxide activity that is usually present in addition to the non-peroxide activity. Sterilization of honey by filtration through microporous membranes is not practicable because of the very high viscosity of honey, but sterilization by gamma-irradiation is a possibility. However, there has been nothing reported on whether or not the antibacterial factors in honey withstand this sterilizing treatment. Therefore this study was undertaken to determine the effect of gamma-irradiation on the antibacterial activity of honey.

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Materials and Methods

Selection of honeys

The honeys used in this study were selected from samples of unpasteurized centrifugally extracted honey obtained from commercial apiarists in New Zealand. These honey samples were collected during the 1994 flowering season. They were chosen on the basis of their antibacterial activity, the manuka honeys having a high level of non-peroxide activity with a low level of hydrogen peroxide activity, the others having a high level of hydrogen peroxide activity only. The honey samples were stored in airtight glass or plastic containers at 10°C in the dark.

The floral sources of the honeys were identified by the suppliers based on colour, aroma, texture and taste of the honey and dominant floral type at the location of the hive at the time of production. The samples used were: Camm. 6B, Comv., Gor. D, B-H, Gor. E., Kint., Rich. – manuka (*Leptospermum scoparium* J. R. et G. Forst., family: *Myrtaceae*); Camm. PS3 – mixed pasture species, primarily clover (*Trifolium repens* L. or *T. pratense* L., family: *Fabaceae*) and nodding thistle (*Carduus nutans* L., family: *Asteraceae*).

Gamma-irradiation of honey samples

All of the samples of honey were sub-sampled into screw-capped 25 mm × 65 mm polyethylene sample vials (30–40 g in each) for processing through a commercial sterilizing plant which guaranteed to subject all items processed to a minimum of 25 kGy of gamma-irradiation. Additional samples of honey were prepared in the same way, then processed through the sterilising plant twice so that they were subjected to a total of 50 kGy of gamma-irradiation.

In order to check that the irradiation was sufficient to kill any clostridial spores which may be present in honey, a sample of honey was heavily seeded with spores of *C. perfringens* and *C. tetani* (10 000 spores of *C. perfringens* together with 1000 spores of *C. tetani* g⁻¹ of honey). These spores were prepared by culturing the bacteria on cooked meat for 3 days at 37°C, then heating the liquid from the cultures at 92°C for 30 min to kill the vegetative cells. The honey used for seeding with spores, Camm. 6B, was selected as having no detectable antibacterial activity when assayed by the method of Allen et al (1991) so as to minimize the possibility of growth of viable spores being inhibited. Samples of the seeded honey (30 g) were then subjected to the same gamma-irradiation procedure as the other samples of honey (25 or 50 kGy of radiation).

After irradiation the seeded honey was tested by the MDL-20 method of Midura et al (1979) for the presence of viable spores, with the enrichment culture being further cultured on blood agar and egg yolk agar plates to aid detection of the bacteria. These plates were inoculated from the enrichment culture daily for 10 days, and incubated anaerobically for 18 h at 37°C. A control plate inoculated with a culture of *C. perfringens* spores was incubated with these plates each day. A sample of the seeded honey that had not been irradiated was also used as a control, this being tested in the same way as the irradiated sample.

In order to determine the sensitivity of the testing technique used, serial dilutions of cultures (in ten-fold dilution steps, in sterile water) of spores of *C. perfringens* and *C. tetani* were used to seed samples of Camm. 6B honey to find the minimum number that could be detected in the honey by the MDL-20 method of Midura et al (1979). The serially diluted cultures were added as 1-mL innocula in the first step of the MDL-20 method, i.e. to the solutions prepared when the 20-g samples of honey were each added to 150 mL of water. For this determination these solutions were sterilised by autoclaving before the inocula were added, so that any vegetative cells or spores present would not give false positive results.

Preparation of honey samples for assay

All samples were prepared aseptically, and were handled away from direct sunlight. A 2-g sample of each honey was weighed out, avoiding any visible pieces of wax. The samples were added to sterile purified water and placed in a 37°C incubator for 30 min to aid mixing by stirring. The 50% (w/v) solutions of each honey sample thus prepared were diluted to 25% (w/v) by taking 1 mL of each and adding it to either 1 mL of sterile purified water or 1 mL of catalase solution. The catalase solution was prepared by adding 40 mg catalase (Sigma, C-10: 4000 units mg⁻¹) to 20 mL of sterile purified water. The samples were assayed immediately after dilution.

Assay of antibacterial activity

The antibacterial activity of the honeys was assayed by the agar well diffusion method. The method was adapted from the punch plate assay for inhibitory substances described in the microbiology standard laboratory methods manual for the New Zealand dairy industry (Anon. 1982).

Large square plates (Nunc Bioassay Dishes, 243 mm × 243 mm × 18 mm) seeded with *Staphylococcus aureus* (ATCC 25923) were prepared by adding 100 µL of an 18-h culture of the bacteria in Trypticase Soy Broth (BBL) to 150 mL of sterilized nutrient agar (made with 8 g L⁻¹ BBL nutrient broth plus 15 g L⁻¹ Difco agar) cooled to 45°C. The plates were poured on a level surface immediately after mixing, and stored for 24 h at 4°C before being used.

Sixty-four wells were cut in the agar with a cooled flamed 8-mm cork borer, using a quasi-Latin square as a template. The template was prepared on a black card, 243 mm × 243 mm. A 25-mm grid was drawn on the card, 34 mm away from the sides, and the wells were centred on the intersections of the grid. The wells were numbered just above the intersections using a quasi-Latin square which enabled the samples to be placed randomly on the plate.

The honey samples were tested by adding 100 µL to each well. One plate was used to test the honey sample from each supplier, with 16 wells being used as replicates for each test. The tests were for total activity before and after irradiation (i.e. 25% w/v honey in water), and for non-peroxide activity before and after irradiation (i.e. 25% w/v honey in catalase solution).

The plates were incubated for 18 h at 37°C and were then placed over the template. Using vernier callipers, the diameter of the clear zones was measured along the horizontal line on the template and recorded in mm. All measurements were recorded without reference to the identity of the samples in the wells.

Results

The antibacterial activity of the honeys before and after gamma-irradiation are shown in a summarized form in Table 1.

Table 1. Comparison of the antibacterial activity of various samples of honey before and after sterilization by gamma-irradiation.

With 25 kGy irradiation Honey sample:	Rich.	Kint.	Comv.	Camm. PS3	zGor. D
Untreated, no catalase	15.39 ± 0.59	16.04 ± 0.84	12.69 ± 0.48	20.82 ± 1.14	13.64 ± 0.53
Irradiated, no catalase	16.32 ± 0.64	14.89 ± 0.56	13.06 ± 0.68	21.25 ± 1.01	14.57 ± 0.47
Untreated, plus catalase	16.32 ± 0.46	0.00 ± 0.00	12.79 ± 0.43	0.00 ± 0.00	12.07 ± 0.55
Irradiated, plus catalase	16.50 ± 0.59	0.00 ± 0.00	13.21 ± 0.43	0.00 ± 0.00	13.54 ± 0.54
With 50 kGy irradiation Honey sample:	Gord. D	B-H	Comv.	Camm. PS3	Gor. E
Untreated, no catalase	14.14 ± 1.10	13.63 ± 1.43	12.06 ± 0.75	18.93 ± 1.59	13.92 ± 1.39
Irradiated, no catalase	15.00 ± 1.70	14.11 ± 1.01	13.08 ± 0.86	18.05 ± 1.79	15.55 ± 1.18
Untreated, plus catalase	13.19 ± 0.73	15.30 ± 1.03	12.09 ± 0.68	0.00 ± 0.00	0.00 ± 0.00
Irradiated, plus catalase	14.51 ± 0.98	14.40 ± 1.11	12.57 ± 0.48	0.00 ± 0.00	0.00 ± 0.00

The activity is shown as the diameter (mm) $\pm$ s.d. (n = 16), of the clear zone obtained in an agar well diffusion assay using plates seeded with *Staphylococcus aureus*. Total activity (i.e. 25% w/v honey in water) and non-peroxide activity (i.e. 25% w/v honey in catalase solution) is shown.

These results show that there was no significant loss of antibacterial activity, of the hydrogen peroxide type or the manuka non-peroxide type, when the honey samples were gamma-irradiated.

In the testing for the presence of clostridia, no viable bacteria were detected in the enrichment culture medium at any stage in the 10-day period of incubation from the irradiated honey seeded with clostridial spores. The control seeded honey (not irradiated) gave heavy growth on the plates after 1 day of incubation in the enrichment culture medium from the unheated inoculum, and after 2 days of incubation in the enrichment culture medium from the heated innoculum.

Growth on the plates was detected from the honey inoculated with serial dilutions after 1-2 days of incubation in the enrichment culture medium with no heating, and after 2-3 days of incubation in the enrichment culture medium after heating, with dilutions down to 11 viable spores mL^{-1} in the inoculum for *C. perfringens* and 5 viable spores mL^{-1} for *C. tetani*. As the 1-mL inoculum was added with 20 g of honey in the test method, this equates to a sensitivity of detection of < 1 spore g^{-1} honey.

Discussion

The possibility of contamination of wounds with microbes from the honey used has not been discussed in any of the reports on the use of honey as a wound dressing. The common reporting of wounds rapidly becoming sterile when dressed with honey suggests that the risk of contamination is likely to be quite low. There is very little literature on the microbial content of honey, but Bentler & Frese (1981) have reported that honey is seldom contaminated with microorganisms, and Tysset et al (1980) have reported that the bacteria present in honey are mostly of the Bacillus genus and non-pathogenic to humans. However, it appears that the spores of *C. botulinum* that are found in honey are not introduced by bees (Huhtanen et al 1981) and so are there presumably as a result of contamination of the honey during extraction and processing. If contamination like this occurs, it raises the possibility of other pathogenic bacteria being introduced into honey. Tysset & Durand (1976) found that various species of *Salmonella* and

Shigella introduced into honey survived from 1.5 months to 2.4 years at 10°C, indicating that such contaminants would survive through the normal storage of commercial honey. Terada et al (1987) found a small proportion of 30 samples of commercial honey to contain *Enterobacteriaceae*, and also found streptococci and staphylococci present. They also found *C. perfringens* in two of the samples. Takayama et al (1987) found *C. perfringens* in 4 of 9 samples of commercial honey.

In view of these reports, together with those cited in the introduction reporting the presence of *C. botulinum* in honey, it could be considered negligent to dress a wound with honey that has not been subjected to a quality-assurance procedure or effective sterilization procedure to guarantee the absence of pathogens. The unreliability of methods for determining the presence of clostridia in honey (Huhtanen et al 1981) makes sterilization the favoured option as long as it does not destroy the antibacterial activity of the honey. The present study shows that honey can be sterilized by a convenient standard procedure that is in common usage for the sterilization of surgical dressings, with full retention of its antibacterial activity even when subjected to twice the normal dosage of radiation.

Although Morgan & Reed (1954) and Bridges & Horne (1959) have found that 30 kGy of gamma-irradiation is needed to sterilize food containing 1-10 *C. botulinum* spores g^{-1}, and 40 kGy for 1000 spores g^{-1}, the testing of the seeded honey in the present study has shown that the 25-kGy dose used in commercial sterilization of surgical dressings, is sufficient to sterilize honey. This honey was very heavily seeded (11 000 spores g^{-1}) relative to the number of spores normally found in honey, yet no viable spores were detected with an enrichment technique which was shown in the present study to allow clostridial spores to be detected in honey down to a concentration of < 1 spore g^{-1} honey. Midura et al (1979) found that the MDL-20 method allowed *C. botulinum* spores to be detected consistently in honey down to a concentration of 7-9 spore g^{-1} honey.

The concentration of clostridial spores occurring in honey normally is much lower than that achieved in the seeded honey. Nakano & Sakaguchi (1991) term 60 spores g^{-1} 'an unusually heavy contamination'. Midura et al (1979) found that in the 90 samples of honey that were examined for *C.*

botulinum spores, eight had 5–25 spores g^{-1} and one had 80 spores g^{-1}. Sugiyama et al (1978) found that of the 241 samples of honey they examined, 18 had spores present, the concentrations being 2–7 per 25 g of honey. Postmes et al (1993) found 40 clostridial spores (*C. perfringens*) per 100 g of honey. They reported that irradiation with 18 kGy rendered the honey sterile without affecting its antibacterial activity.

The high sugar content of honey, its acidity and the presence of other antibacterial factors, make it unlikely that clostridia will multiply to give large numbers in honey: Nakano & Sakaguchi (1991) found that not only did *C. botulinum* not grow in honey, undiluted and diluted 1:2, but also that on incubation the number of spores decreased. This inhibitory action of honey would also reduce the chance of toxins being produced in the honey.

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