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The Effects of Honey Compared With Sucrose and a Sugar-free Diet on Neutrophil Phagocytosis and Lymphocyte Numbers after Long-term Feeding in Rats

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Abstract

To determine whether honey and sucrose would have differential effects on levels of neutrophil phagocytosis after long-term feeding 36 2-month old Sprague Dawley rats were fed a powdered diet that was either sugar-free or contained 7.9% sucrose or 10% honey (honey is 21% water) *ad libitum* for 52 weeks. The percent of neutrophils exhibiting phagocytosis, and the percentage of leukocytes that were lymphocytes were then measured by flow cytometry after 52 weeks. Results: Neutrophil phagocytosis was similar between sucrose- and honey-fed rats, and lower in rats fed the sugar-free diet (79.2%, 74.7% and 51.7 %, respectively). The percentage of leukocytes that were lymphocytes differed significantly between all three treatments, the levels being highest in honey-fed rats (53% vs 40.1% and 29.5% for sucrose- and sugar-free fed rats). In conclusion: Honey may have a beneficial effect on immune activity, possibly attenuating the decline seen in older age.

KEYWORDS: Honey, sucrose, neutrophil phagocytosis, immunity, lymphocytes

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INTRODUCTION

Immune function is known to decline in older age and limited data suggests that this is due to dysregulation of the immune system rather than a decrease in immune response (Lesourd, 1997). In particular, aging in humans is associated with a decrease in cell-mediated immunity including a reduction in peripheral blood lymphocyte counts, and with changes in T lymphocyte subpopulations (Lesourd, 1997; Castle, 2000). Similarly, neutrophil activity has also been shown to decline in older populations (Wenisch et al. 2000; Butcher et al. 2001). The latter may be of particular importance as neutrophils are considered to be the "first line of defense", these being the leukocytes that respond most rapidly to a pathogenic invasion. Research has shown that prebiotics have the ability to enhance immune responses (Yamada et al. 1999; Schley and Field, 2002) and anecdotal evidence suggests that honey can stimulate immune function. Limited research has demonstrated that honey can stimulate the release of tumour necrosis factor-α •and cytokines in monocyte cells (Tonks et al., 2001, 2003), and increase proliferation of B- and T-lymphocytes and neutrophils *in vitro* (Abuharfeil et al. 1999). In addition, honey has been demonstrated to stimulate antibody production during primary and secondary immune responses against thymus-dependent and thymus-independent antigens (Al-Waili and Haq, 2004). This trial was therefore designed to ascertain what effect honey might have on the immunity levels of older rats, compared with those fed a sucrose-based or sugar-free diet, after long term feeding.

MATERIALS AND METHODS

Experimental Animals and Housing

This study included 36 male Sprague Dawley rats, aged approximately 8 weeks at the start of the trial, sourced from the small animal research facility at AgResearch, Hamilton, New Zealand. The trial was carried out in the Behaviour Research Facility at Waikato University, Hamilton, New Zealand.

The research room was maintained at 22±1°C with a 12 hour light/dark cycle (lights off 0700h). This study was approved by the Waikato University Animal Ethics Committee.

Diets

Three experimental diets were prepared to contain either no sugar (diet # 1), 7.9% sucrose (diet # 2) or 10% Honeydew honey (diet # 3) (the honey was 21% water) as well as the following (g/kg diet): skim milk powder, 95; casein, 120; oil, 160; cellulose, 50; mineral mix, 50, sugar-free vitamin mix, 5, starch, 365. The diets were prepared such that they were equivalent to a typical New Zealand diet, based upon data from the 1997 National Nutrition Survey (1999). Of 100% total energy, 15–16% came from protein, 35% came from fat, and 45–47% came from carbohydrate. Low GI amylose was used in place of sucrose and honey in the sugar-free diet.

Diets were prepared monthly and were kept in the dark at 4°C or -15°C for the duration of the study. Standard rodent vitamin and mineral mixes was prepared as described previously (Chepulis, 2007).

Experimental Design

All animals were weighed upon receipt, randomly allocated to one of three experimental diets and housed individually in standard rat cages (45 cm x 25 cm x 30 cm high) with plastic bottoms and metal grid lids. Food jars containing the appropriate diet were placed into the respective cages and new diet added to the jars every two days so that food was available *ad libitum*. Water was freely available, and replaced twice weekly. All animals were weighed every 1–2 weeks.

On days 364 and 365 (half of each treatment group on each day), the rats were anaethetised using CO_2 gas. Each animal underwent a cervical dislocation before a sample of blood was removed from the heart via cardiac puncture using 19 gauge needles. The samples were taken to AgResearch, Hamilton, New Zealand (within 2 hours of collection) and assayed using Flow cytometry as follows. Briefly, 100 µl of whole blood was added to pre-cooled FACS tubes in an ice bath. A sample (50 µl) of fluorescein isothiocyanate (FITC)-labelled *E.coli* (prepared in-house using non-oponised K12 strain *E.coli* and Fluorescein conjugate W-2861) containing 4 x 10^6 bacteria was then added to each tube (except the control) and the tubes incubated at 37°C for 10 minutes (the control tube was left in the icebath). After incubation, 50 µl of FITC-labelled *E.coli* was added to the control tube. All tubes were then lysed with FACS lyse buffer (BD Biosciences Catalog number 349202) and the tubes incubated at room temperature in the dark for 10 minutes. The tubes were then centrifuged at 1200 rpm for 10 minutes at 18°C, the supernatant discarded and the pellets re-suspended in 1mL each of PBS buffer. The tubes were again centrifuged as described and the supernatant discarded. Each sample was then re-suspended in 500 µl running buffer (1% paraformaldehyde in PBS), and 50 µl Trypan blue (0.4% in PBS) added to each tube. These samples were then run on a Becton Dickenson Flow Cytometer and the percent neutrophil phagocytosis and percent leukocytes that were lymphocytes determined.

Statistical Analyses

All values are expressed as mean ± SD. A one-way analysis of variance (ANOVA) was performed on all parameters to determine if there were statistical differences between the three dietary treatments.

RESULTS

All animals appeared healthy throughout the 52-week study and no adverse events were recorded. The results are presented in Table 1. Mean overall percentage weight gain in honey-fed rats was similar to that observed in rats fed a sugar-free diet, and was significantly reduced compared with sucrose-fed rats after 52 weeks ($p = 0.015$). Mean overall percentage weight

gain was also significantly reduced in rats fed the sugar-free diet compared with the sucrose-fed rats ($p = 0.004$). Food intake did not differ between the three dietary treatments throughout the 12 month period (see Chepulis and Starkey, 2007 for further details).

The percentage of neutrophils that exhibited fluorescent phagocytosis was similar between sucrose- and honey-fed rats, and both of these treatments were significantly higher than rats fed the sugar-free diet (both $p < 0.0001$). The percentage of leukocytes that were lymphocytes was nearly twice as high in honey-fed rats as in those fed the sugar free diet ($p < 0.0001$), and nearly 13% higher than the sucrose-fed rats ($p = 0.002$). The percentage lymphocyte values for rats fed the sucrose and sugar-free diets were significantly different at $p < 0.0001$.

Table 1: Endpoint Data (mean ± SD) for rats fed diets that were either sugar-free, or contained 7.9% sucrose or 10% honey for 12 months.

Result	Unit	Diet		
		Sugar-free (Diet # 1)	**Sucrose (Diet # 2)**	**Honey (Diet # 3)**
Overall weight gain	%	102.5 ± 19.7†	130.6 ± 26.7*	107.2 ± 13.8 †
Overall food intake[a]	g/7 w	1246.4 ± 85	1243.6 ± 111	1244.8 ± 89
Overall kilojoule intake	kJ/7 w	23182 ± 1580	23019 ± 2053	22730 ± 1620
Neutrophil phagocytosis	%	51.7 ± 11.7†‡	79.2 ± 11.5*	74.7 ± 14.6*
% lymphocytes[b]	%	29.5 ± 8.0†‡	40.1 ± 10.8*‡	53.0 ± 6.6*†

* significant *vs* sugar-free diet; † significant *vs* sucrose diet; ‡significant vs honey diet (p values given in the text)

[a] Food intake was assessed during 7 week-long periods, each two months apart. Animals were placed into mesh-bottom cages and the amount of consumed diet recorded. A 4-day lead-in period was used prior to assessing food intake to allow the animals to acclimitise to the different cages. Food intake was corrected for percentage dry matter (determined by heating duplicate samples for 16 hours at 105°C)

[b] Percentage of leukocytes that were lymphocytes. The data was generated by the flow cytometer; actual counts were not generated.

DISCUSSION

Long-term feeding of honey has been shown in our laboratory to have a number of health benefits, including improved weight regulation, decreased oxidative damage and improved cognitive functions compared with rats fed sucrose or a sugar-free diet (Chepulis and Starkey, 2007; Chepulis et al., 2007) and it appears that honey may also stimulate immune function in aged rats. Neutrophils mediate the immediate host response to bacterial and fungal

infections, which are largely responsible for the higher rates of mortality and morbidity seen in older populations (Butcher et al. 2001). In human populations, total neutrophil numbers are not thought to decline; however, a decrease in neutrophil function (phagocytic activity) has been shown to occur with increasing age (Wenisch et al. 2000; Butcher et al. 2001).

In this study, the degree of neutrophil phagocytosis was similar in both honey- and sucrose-fed rats (75–80%), both of these treatments having approximately 50% higher levels of phagocytosis than rats fed the sugar-free diet. Many other studies have investigated neutrophil function in rats, but given that every trial used different diets, breeds, age and drug interventions it is impossible to get comparable data. Thus, it is impossible to know whether the higher levels of neutrophil phagocytosis in honey- and sucrose-fed rats reflect a reduced decline with age, or whether there has been a stimulation of phagocytic activity in these two groups that did not occur in those fed the sugar-free diet. A review of the literature revealed nothing that might suggest that sucrose could affect the activity of the immune system, although there is some limited data available for the effects of feeding honey on total neutrophil numbers. In one small study (Al-Waili, 2004) 12 sheep (6–8 months; 25–30 kg) were randomized to receive either 500 ml of intravenous honey (5% w/v) or saline once every 10 days for 40 days, with blood removed at 0, 10, 20, 30, 40 and 50 days. The % neutrophils (it is not stated, but the reader must assume that the author mean the percent of leukocytes that were neutrophils) increased from 55% at time zero to 76% in the honey group after 10 days, whereas no difference was observed in the saline group. In the present study, no actual neutrophil counts were measured, but it is possible that the increased phagocytosis seen in honey- and sucrose-fed rats resulted from an increase in the number of new (and maybe more active) neutrophils. This idea is supported by the finding that the percentage of leukocytes that were lymphocytes was higher in honey- and sucrose-fed rats compared with those given the sugar-free diet (and also higher in honey-fed rats compared to those fed sucrose). Ideally, it would have been best to have measured the levels of neutrophil phagocytosis and lymphocyte counts throughout the 12 months, rather than just at the end of the study, so as to get a better idea of the trend of phagocytic activity over time; however, this was not practical in this study due to financial constraints. It would certainly be advantageous, though, to investigate these trends in lymphocyte counts and neutrophil phagocytosis in future feeding studies.

The findings from this research do demonstrate, though, that honey may have a better immuno-modulatory effect than sucrose alone, as honey-fed rats had percentage lymphocyte counts that were 1.3 times higher than those of the sucrose-fed animals. The honey used in this study contained 40.9% fructose, 30.1% glucose, 0.5% sucrose, 3.4% maltose and 5.1% oligosaccharides (Chepulis, 2007), these levels being comparable to the average values reported elsewhere (White, 1975; Rousseau et al. 1980). As sucrose is a dimer of glucose:fructose, the dietary concentrations of fructose and glucose resulting from the intake of the sucrose diet would both have been

3.95g / 100g diet. This is comparable to the 4.09g / 100g fructose and 3.01g /100g glucose consumed in the honey diet (4.34g and 3.26g /100g, respectively, when the sucrose present in honey is taken into account), suggesting that the sugar content of the two diets does not explain the difference in lymphocyte counts, although it is possible that the small differences in sugar concentrations may have been involved. Indeed, honey has been shown to have a mitogenic effect on human B and T lymphocytes (Abuharfeil et al. 1999) and it has been suggested that this may occur through the glycosylation of proteins resulting from the high sugar content (glycosylated proteins have been shown to activate a number of cell types).

Research has also demonstrated that sugars that are slowly absorbed can lead to the formation of short chain fatty acid (SCFA) fermentation products in the gut (Kruse, 1999). SCFA are commonly formed after ingestion of pre-biotic foods, and with the pre-biotic effects of honey established *in vitro* (Ustunol and Ghandi, 2001; Sanz et al. 2005) it is possible that the ingestion of honey may also result in their formation. A number of studies have demonstrated, either directly or indirectly, that SCFA can have immunomodulatory properties (reviewed by Schley and Field, 2002). Thus it is possible that honey may also be stimulating the immune system via the presence of fermentable sugars in the gut.

It is also possible that non-sugar components of the honey may be responsible for the enhanced immuno-modulatory effects seen. Tonks et al. (2007), for example, have recently reported that a 5.8 kDa component of Manuka honey is capable of stimulating immune function *in vitro*. These authors determined that the compound was not a lipopolysaccharide, amino acid, vitamin or mineral, but further investigations are required to elucidate the nature of the compound. It is also possible that the antioxidant content of the honey may have contributed to its immuno-modulatory effects. Although there are no studies directly investigating the effects of honey antioxidants on immune function, other antioxidant compounds have been shown to stimulate immune function *in vitro* (Sabongi et al. 1997) and *in vivo* (De La Fuente et al. 2002). However, further investigations, perhaps using artificial immune challenges, are recommended to better elucidate these effects.

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