

# An *in vitro* evaluation of the cell toxicity of honey and silver dressings

- **Objective:** To establish whether honey and silver-impregnated dressings used by wound-healing practitioners are cytotoxic *in vitro* to human skin keratinocytes and dermal fibroblasts.
- **Method:** Human keratinocyte and fibroblast tissue cultures were established *in vitro*. Untreated cultures served as controls (group 1). Small dressing implants of monofloral, medicinal honey (L-Mesitran) (group 2) and nanocrystalline silver (Acticoat) (group 3) were placed in test wells and co-cultured with each of the two cell lines. Morphological changes, including cell toxicity, were assessed using inverted microscopy, trypan blue staining and the Rosdy and Clauss cell toxicity scoring system.
- **Results:** Untreated cultures consisting of both keratinocytes and fibroblasts (group 1) were established in 90% of all cases. In group 2, cultures with honey-impregnated implants, cell proliferation remained present at two and four months. Cell viability remained intact and cell toxicity was not evident at four months after continuous tissue culture. In group 3, marked toxicity was observed with high non-viability staining and cell-scoring counts compared with groups 1 and 2 ( $p < 0.05$ ). This demonstrates that the silver interfered with epidermal cell proliferation and migration, implying that it contains cytotoxic material.
- **Conclusion:** The honey-based product showed excellent cytocompatibility with tissue cell cultures compared with the silver dressing, which demonstrated consistent culture and cell toxicity. Further studies are needed to assess if these comparative *in-vitro* findings should influence a clinician's choice of wound dressing.
- **Declaration of interest:** None.

honey; silver; tissue culture; keratinocyte; cytotoxicity testing; fibroblast

The potential advantages and disadvantages of silver or honey dressings in chronic wound healing have been well documented,<sup>1-5</sup> and their antimicrobial benefits are not disputed.

Honey's healing efficacy is partially attributed to its antimicrobial and anti-inflammatory properties, its ability to deodorise malodorous wounds and promote autolytic debridement and a moist wound-healing environment.<sup>2,4</sup> However, the precise mechanism of action of honey in advancing wound healing at the cellular level is still under investigation, while silver dressings can have mild to severe negative cell cytopathic properties.<sup>6-8</sup>

The published evidence for the effects of various honey and silver dressings on tissue-cultured keratinocyte and fibroblast explants is inconclusive.<sup>3,5</sup> This study was undertaken to provide new data and insight in this regard. It is one of a small number of tissue-culture cell-biology investigations that compare the potential cell toxicity of honey and silver dressings *in vitro*.

Human-derived epidermal keratinocytes and dermal fibroblasts were selected for testing because they are key cellular components of the wound-healing cascade and repair process.<sup>9-13</sup>

## Method

An organic, monofloral, hydroactive, medicinal-grade honey dressing (L-Mesitran Hydro, Triticum, the Netherlands) and a nanocrystalline silver-impregnated dressing (Acticoat, Smith & Nephew) were selected for this *in-vitro* study. They are considered to represent the honey and silver preparations used by practitioners. The silver dressing served as a culture control to the honey product.

Primary outcomes were to compare the effects of the honey and silver dressings on epidermal and dermal cell proliferation and to document any negative growth (that is, if one or both compounds have a negative effect on cell growth) and local cell toxicity under standard tissue culture conditions.

Cell proliferation was studied within an ethical authorised tissue-culture research protocol for utilising keratinocytes and fibroblasts.

## Development of cell culture

Human-derived epidermal keratinocytes and fibroblasts were selected for testing. To gain statistical power and allow for cell biological variation and failures, approximately two million cultured cells of each cell line were randomised into 144 tissue-culture test wells.

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For each cell line, 36 wells were utilised and 1,048 tissue explants (2 x 2mm) were inserted. Tissue explants were carefully prepared aseptically and fragmented into small blocks on a glass surface by sharp dissection under magnification of a dissection microscope (Olympus, Japan). Between five and 10 explants were arranged on the periphery of each well under magnification.

Of the 144 wells, 108 were used as follows: keratinocytes (n=36), fibroblasts (n=36) and control testing (n= 36 x 2 were used for media controls). Post-culture validation revealed 92–98.5% viability of explant-generated cells. Initial cell counts in the first 14-days varied from 158,000 to 1.5 million. The highest count recorded on day 21 post-culture was 12.5 million.

Conventional cell-culture methodology was used in a sterile tissue-tissue culture facility. Basic culture medium (Dulbecco's Modified Eagle's Medium, Highveld Biologicals, South Africa) was enriched with antibiotics (penicillin, streptomycin and amphotericin-B). Tissue plating and media exchanges were conducted in a conventional laminar flow hood. Cell cultures were exposed to both serum-enriched and serum-free control media. Cultures were put up in triplicate on fibronectin-based Petri dishes. Culturing was affected at 37°C in a carbon dioxide incubator (Shell Lab). The source of tissue explants was donated human skin.

The two cell lines were each divided into three groups:

- *Group 1* — control tissue culture with cellular explants, to which no product would be added
- *Group 2* — tissue culture, to which the honey product (L Mesitran) would be added
- *Group 3* — tissue culture, to which the silver product (Acticoat) would be added.

#### Insertion of the test products

Five and 21 days after initiating the tissue culture, the honey or silver product was introduced directly onto the cells in the test wells to allow cell growth and release from the tissue explants to occur immediately adjacent to and around the product implant. Using fine-toothed forceps, small, sterile blocks of the commercial dressings of uniform size (2 x 2mm) were therefore inserted into the Petri dishes adjacent and distal to the tissue explants. The biological effects on cell growth were carefully assessed.

#### Cell testing and cell statistics

Primary and secondary cultures were established and culture media were exchanged three times a week under rigid, controlled, aseptic conditions, using conventional methodology.<sup>11</sup> Cell proliferation and cell morphology criteria assessed in culture included locomotion (crawling), filopodia formation (cell outgrowths), branching, network forma-

tion, cell death and monolayer dysfunction. These were captured daily over a four-month period using inverted light microscopy.<sup>11</sup> Cell viability was determined randomly using trypan blue or crystal violet staining.<sup>11</sup> To avoid contamination, these tests were reduced to a minimum. Cell audits, record keeping and cell tracking were carefully recorded in cell-testing registries. All cell lines were discarded at the end of the study.

Early cell proliferation of both keratinocytes and fibroblasts from the tissue explants was visible in the majority of test wells on days 5–7. Each of the two test products was exposed to each of the two cell lines at different phases of cellular growth: single rapidly dividing cells and established monolayers.

Cell morphology parameters and histological criteria were based on standard works in histology and cell biology.<sup>11-13</sup>

Parametric and non-parametric statistical analysis to assess cell proliferation included the Student's t-test or Mann Whitney U Test for small numbers (Statgraphics). Visual images were captured on a digital camera (Sony/Zeiss, Japan).

#### Cell biology assessment after product stimulation

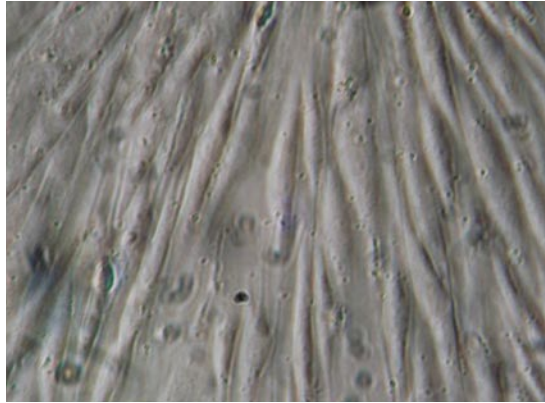
Cell morphology, including cell shape changes, was assessed daily under an inverted microscope (Olympus CKX31) to evaluate nuclear and gross cytocavitary morphology (cell nucleus and cytoplasm characteristics), thus monitoring:

- Cell proliferation and migration
- Cell toxicity
- Killing or inhibitory effects of the test products
- Cytocompatibility or cytopathic effects.<sup>11</sup>

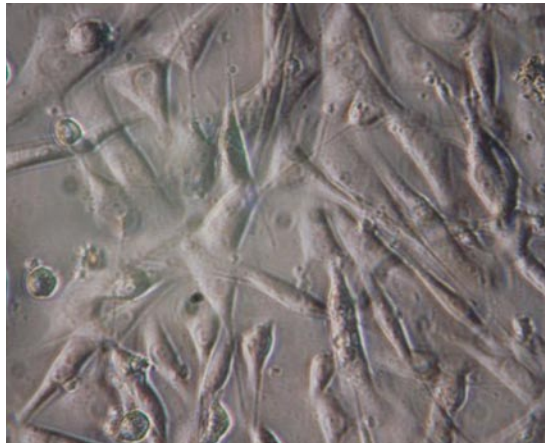
Cellular viability was performed on an ad hoc basis by the application of the exclusion trypan blue or crystal violet tests. Cytokine, dressing absorbency tests, cell extractions, flow cytometry and yellow dye test assays were beyond the scope of the study. For each experiment, the tests were performed in duplicate.

Cell shape and configuration of keratinocytes and fibroblasts were evaluated by inverted microscopy using the Rosdy and Clauss scoring system for comparison of the effects of the two products on cells in the test wells.<sup>10</sup> This permitted appropriate cell testing, measurement and scoring of cell proliferation and morphological changes affecting cell shape as follows:

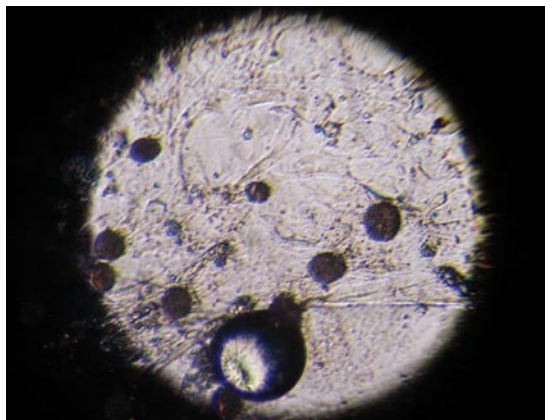
- 0 = No change noted
- 1 = Slight, but clear change in shape without decrease of cell density
- 2 = Considerable alterations in cell shape and slight decrease of cell density
- 3 = Loss of cell shape. More than 50% of cells detached
- 4 = All the cells are dead, lysed or detached.



**Fig 1. Group 1 (control):** photomicrograph showing approximately 50 fibroblasts per high-field, reflecting normal cellular morphology, proliferation and expansion in monolayer culture; cell-scoring count of zero



**Fig 2. Group 2 (honey):** photomicrograph image showing approximately sixty intact branching and dividing fibroblasts per high-field in the presence of honey with a cell-scoring count of zero



**Fig 3. Group 3 (silver):** photomicrograph image showing severe silver-induced inhibition of cell-expansion and cell-death with a cell-scoring-count of 3–4. Approximately 20 abnormal fibroblasts per high-field are visible

## Results

### Group 1: untreated cells

Cell proliferation of epidermal keratinocytes and dermal fibroblasts was prospectively established in more than 90% per cell line at one week after the insertion of the tissue explant into test wells. Keratinocytes proliferated slightly faster than fibroblasts and appeared earlier from the tissue explant edges, usually on days 4–5, except in serum-free media where erratic proliferation was detected, and then increased exponentially. With time, fibroblast growth increased and exceeded that of keratinocytes. Cell proliferation was observed in 95% of tissue explants in the absence of contamination or infection. A primary culture failure rate of 10% was observed and is consistent with retrospective findings in this laboratory.

Fig 1 shows normal fibroblast proliferation in control group I during cell testing. Monolayers were usually established within three weeks, but serum-free media rendered the poorest results. Cultures were established until four months, at which stage cell degeneration due to senescence was evident in both cell lines. Cell scoring was zero, which is consistent with normal cell proliferation throughout the observation period. Trypan blue stains were in the range for normal controls.

### Group 2: exposure to the honey product

For both keratinocytes and fibroblasts, there was a modest uniform increase in early cell proliferation and cell counts per millimetre in most of the wells containing honey implants, when compared with the untreated controls (group 1) and silver product (group 3), based on the assessed criteria and parameters. This became more apparent as monolayers developed during cell testing. Nuclear and cytoplasmic networks appeared normal, suggesting no local toxicity. Cell proliferation was also evident immediately adjacent to the product, suggesting no local toxicity.

Disappointing cell stimulation and proliferation for both keratinocytes and fibroblasts were detected in serum-free wells, confirming our previous experience with explant cell expansion (group 1). Honey implants significantly stimulated single cell and monolayer formation of both keratinocytes and fibroblasts and branching of fibroblasts, when compared with cultures in serum-free medium.

Honey implants were not toxic to early cell proliferation, nor did they affect established monolayers of both keratinocytes and fibroblasts. This indicates epidermal cytocompatibility. Cell crawling and locomotion were enhanced, especially when monolayers were established.

Primary culture failure in both keratinocyte and fibroblast lines was 10%, the same as in the control group (group 1,  $p > 0.05$ ), and reflects our previous experience.

Keratinocytes and fibroblasts continued to proliferate.

erate strongly at four months and after three passages (cell medium changes in dishes).

Fig 2 shows intact fibroblasts cultured in the presence of honey without signs of morphological toxicity. Trypan blue studies showed the presence of a very small percentage of non-viable cells, but this was not significant when compared with the untreated controls (group I:  $p > 0.05$ ). Cell scoring remained at zero for more than two months and did not differ from untreated controls (group I,  $p = \text{NS}$ ).

### Group 3: exposure to silver product

Poor keratinocyte and fibroblast cell proliferation and cell monolayer formation was detected uniformly in the silver-treated wells when compared with honey implants (group 2) and untreated controls (group 1). The culture failure was 40% ( $p < 0.05$ ). Killing and inhibitory effects of silver on cell growth throughout both cell lines were rapidly evident during cell testing.

Enhanced cell proliferation was not detectable, when compared with honey (group 2,  $p < 0.05$ ). Cell survival, migration and shape were negatively affected. General single-cell proliferation and monolayers were inhibited in the silver group compared with honey (group 2), and cell numbers declined with time, indicating continuous and ongoing cell toxicity (groups 1 and 2,  $p < 0.05$ ). In some wells, enhanced cell failure was prominent and monolayers never formed. Despite the small sample size, striking negative morphological changes, including cell proliferation and monolayer inhibition, were detectable in this group. Silver-treated cells and tissue explants fared poorly in culture, and showed poor proliferation potential compared with honey-treated wells. Trypan blue staining showed a high percentage of non-viable cells compared with groups 1 and 2 ( $p < 0.05$ ).

Fig 3 shows poor cell proliferation in the presence of the silver product, and is representative of ongoing cell inhibition as seen in this group. There were no surviving keratinocyte or fibroblast cultures or monolayers at three weeks ( $p < 0.05$ , versus group 1 or 2). Trypan blue staining confirmed high counts, indicating that non-viable cells and cell-shape changes ranged from 3-4 (80%  $> 3$ , groups 1 and 2,  $p < 0.05$ ). This indicates cell proliferation inhibition and cytopathic effects. Very poor cell counts, of both cell lines, were recorded.

### Discussion

These findings demonstrate that honey is a modest cell stimulator of early proliferation of both types of cell *in vitro*. The honey-based product placed in test wells was clearly superior in this respect when compared with the silver-impregnated dressing. After one month of continuous stimulation and exposure, there was no apparent over-proliferation of

cells treated with the honey product compared with the control cultures. The results confirm other reports that silver-impregnated dressings are potentially cytopathic and cytotoxic to sensitive proliferating cells *in vitro*.<sup>5-8</sup> These observations may be relevant *in vivo* and in clinical decision-making.

The mechanism by which honey enhances cell stimulation *in vitro* is still open to speculation. However, the findings may imply that honey-impregnated dressings should be used with caution in the immediate vicinity of malignant ulcers until the safety of their use for this purpose is proven. This study did not address the antimicrobial spectrum of the two products, or of different types of honey product, but it is evident that silver-based wound dressings are cytotoxic to fibroblasts and keratinocytes. This was detectable in the majority of test wells treated with the silver dressing and confirms other anecdotal reports.<sup>5-8</sup>

### Honey-based dressings

This study has established that the selected honey-impregnated dressing could modestly stimulate human epidermal and dermal cells *in vitro*. However, we are unable to comment on this dressing's effect on collagen and elastin deposition, or formation of the extracellular matrix. Other investigators have also reported favourable wound-healing properties of honey preparations in both laboratory and clinic.<sup>3,14-16</sup> A Cochrane analysis indicates that honey wound dressings and formulations may improve healing times in mild to moderate superficial and partial-thickness burns, when compared with standard care.<sup>15</sup> Others have demonstrated that topical application of honey products to split-thickness skin-graft donor sites speeds up epithelialisation or re-epithelialisation, suggesting that it may enhance the spread and outgrowth of keratinocytes from hair follicles in the donor site.<sup>17</sup>

Honey, a viscous, super-saturated sugar solution, is widely used in various wound-care formulations.<sup>3,16,17</sup> There is much speculation, and some evidence from animal studies and small clinical trials, that it may modestly accelerate wound healing but the precise mechanisms remain obscure.<sup>3</sup> In the case of acute wounds, honey dressings may well reduce healing times in patients with partial-thickness burns.<sup>17</sup> However, in chronic wounds, such as venous leg ulcers, honey dressings had a poor outcome compared with 'standard wound care' and compression, and did not increase wound healing.<sup>18</sup> In the clinical setting, honey dressings do not always improve wound healing over standard care.<sup>18</sup> However, this study provides additional laboratory and biomedical evidence that the selected honey dressing can modestly enhance cell proliferation and expansion *in vitro*, compared with control cultures. It also suggests it is non-toxic to cell cultures, when

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compared with the silver comparator.

Other workers have referred to the unspecified potential biological wound-healing properties of honey preparations, and highlighted its antimicrobial potential.<sup>2,3,19</sup> Researchers have tentatively speculated on how honey preparations may influence the delicate wound-healing cascade. Researchers from Wales have shown that it can stimulate inflammatory cytokine production from monocytes,<sup>19</sup> and have indicated that a range of honey derivatives, including manuka and pasture honey, increase the release of tumour necrosis factor-2, interleukin-1 beta (IL1 $\beta$ ) and interleukin-6 (IL6) from MM6 macrophage cells and human monocytes. These processes play subtle roles in cell signalling and wound healing, including fibroblast proliferation, angiogenesis and collagen synthesis.<sup>19-22</sup> IL6 is mitogenic for keratinocytes, and so may contribute to epithelialisation.<sup>22</sup>

TNF-alpha also induces IL6 production by keratinocytes.<sup>19</sup> TNF-alpha and IL1b potentially stimulate platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-beta (also found in platelet-rich plasma), both of which are chemotactic for monocytes and fibroblasts and maintain the activity of these cell elements within a wound and in the extracellular matrix.<sup>20,23</sup>

Animal studies conducted at the University of Malaya suggest that honey accelerates wound healing by influencing cell division, proliferation, collagen synthesis, wound retraction and epithelialisation.<sup>14</sup> Suguna et al. have reported favourable effects of honey on glucosamine synthesis.<sup>24</sup> They confirm that honey can accelerate the synthesis and maturation of collagen, resulting in increased tensile strength in skin wounds.<sup>24</sup> Their work is supported by the observations of Rozaini et al.<sup>16</sup> Work from the University of Kentucky shows that honey can provide energy for the cellular activity needed in the wound-healing process.<sup>25</sup>

Our study confirms that monofloral, medicinal honey, used alone and in the absence of monocytes, enhances proliferation of keratinocytes and fibroblasts, but the initiating factors remain unknown. We speculate that the fibronectin tissue-coated plates may initiate or 'kick start' proliferation of these cells and that the following rapid proliferation may well be due to an autocrine effect, induced by the local release of interleukins, cytokines, prostaglandins or combinations of these from the proliferating cells in contact with the honey.<sup>26,27</sup>

Enhanced wound contraction in fresh wounds dressed with honey has been demonstrated recently in Wistar rats by Nigerian researchers.<sup>27</sup> Myofibroblasts play a key role here, and this work may indicate that topically applied honey stimulates myofibroblasts to enhance wound healing.<sup>27,28</sup>

Fibroblasts have diverse functions, such as deposi-

tion of collagen and elastin scaffolding in the extracellular matrix and maintenance of tissue cohesion, and they possibly also function as antigen-presenting cells.<sup>14</sup> In the bone marrow micro environment, fibroblasts are very responsive to IL1-alpha stimulation and are associated with the release of biologically active haemopoietic growth factors.<sup>29</sup> Dermal fibroblasts can also release granulocyte macrophage-colony stimulating factors (GMCSFs), granulocyte-colony stimulating factor (GCSF) and macrophage-colony stimulating factor (MCSF) in tissue culture, all of which play an important role in the haemopoietic response to inflammation.<sup>29</sup> These important observations suggest, in part, that different types and grades of honey may stimulate inflammatory cytokines from monocytic and other cells known to play an important role in wound healing and tissue repair.<sup>22</sup> There is therefore direct and indirect supporting data from other centres that honey may enhance stimulation of epidermal and dermal components with a predictable effect on the extracellular matrix.

**Silver-impregnated dressings**

Silver-impregnated dressings are used extensively by practitioners in the care of chronic wounds, presumably because of their documented antimicrobial properties.<sup>5,8,30</sup> However, carefully controlled cell biology studies have demonstrated significant evidence of cytotoxicity regarding topical silver and silver-impregnated dressings,<sup>30</sup> including the application of topical 1% silver sulphadiazine (Silvasone, Flamazine) and silver-based dressings (Acticoat).

Other *in-vitro* culture studies show evidence of keratinocyte cytotoxicity following exposure to silver,<sup>30,31</sup> an observation which was confirmed in this study. Although these independent studies do not provide evidence of superiority or inferiority for any particular silver product, the findings of cell cytopathogenicity should not be ignored by practitioners.<sup>5</sup>

Researchers from Australia report similar cell toxicity outcomes in other silver-containing dressings (Aquacell AG, Avance and Contreet H)<sup>32</sup> and suggest that these findings imply that rapidly proliferating cells (such as in donor graft sites, superficial burns and following keratinocyte transplantation) are very sensitive and risk potential cytotoxicity if exposed to a silver dressing product.<sup>32</sup> They recommend that silver-based dressings are potentially cytotoxic, so should be avoided when treating wounds in the absence of infection.<sup>32</sup>

Earlier cytotoxic studies conducted by independent French researchers focusing on burn management showed no apparent toxicity towards fibroblasts and epidermal cells when exposed to other conventional or non-silver-impregnated wound dressings such as Tegaderm or Jelonet.<sup>10</sup> A recent study from China<sup>5</sup> also confirms silver-induced toxic-

ity in cell monolayers in tissue culture and tissue explant cultures that have potential clinical wound-healing implications, including inhibition of proliferation and expansion of keratinocytes and fibroblasts with resulting wound-healing retardation or deficient re-epithelialisation. Clinically, this could manifest as impaired re-epithelialisation of donor skin sites, delayed wound healing or inhibition of wound epithelialisation. Our study also confirms the toxicity of the silver product with respect to keratinocytes and fibroblasts when exposed to a silver derivative in tissue culture. The quantification and verification data reported here show inhibition of cell migration and stimulation together with silver-induced cell toxicity.

Cell culture testing is a reasonably sensitive and reproducible instrument to assess silver toxicity.<sup>6,10,31-34</sup> However, our 'cell-based' arguments in favour of the use of honey dressings may be only of theoretical interest, especially in situations where dressing choice is critical and wound bioburden is high, when a silver dressing may be the preferred choice. This study is not intended to prove or disprove the claims of any commercial sponsored research,<sup>30</sup> nor does it endorse or criticise specific dressings. It may well be that the antimicrobial effects of silver dressings far outweigh the disadvantage of cell toxicity and potential retardation of wound-healing elements shown *in-vitro* here and elsewhere.<sup>30,32</sup>

In the clinical situation, the bacterial 'killing

zone' of silver may be restricted to the inside of a dressing, rather than being in contact with the wound, thus avoiding 'collateral damage' to healthy keratinocytes and fibroblasts within and around the wound.<sup>5</sup> A hydrogel device may be a wise clinical choice, because the exudate is drawn up into the dressing providing for exudate retention or more effective control of toxic exudate. However, the multi-purpose honey-based product used in this *in vitro* study has definitive advantages over silver dressings, especially when wound healing is the objective. This is because the unique cell biological characteristics of honey favour keratinocyte and fibroblast proliferation, which play a key role in re-epithelialisation and re-establishment of the extracellular matrix.<sup>9,27</sup>

### Conclusion

We have examined the cell morphological effects of two key cellular components of wound healing, keratinocytes and fibroblasts, when in contact with honey- and silver-impregnated dressings in a tissue explant, culture model *in vitro*. Honey-impregnated dressings have the potential to promote new tissue regeneration or healing, and there is evidence to support this.<sup>15</sup>

The robust science outcomes of this study lead us to support the continued use of honey-impregnated dressings by wound-care practitioners, in preference to a silver-based equivalent. ■

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