Activation of AMPK/Nrf2 signalling by Manuka honey protects human dermal fibroblasts against oxidative damage by improving antioxidant response and mitochondrial function promoting wound healing

José Miguel Alvarez-Suarez a,b, Francesca Giampieri a,* , Mario Cordero c, Massimiliano Gasparrini a, Tamara Yuliet Forbes-Hernández a,d, Luca Mazzoni e, Sadia Afrin a, Pablo Beltrán-Ayala f, Ana María González-Paramás g, Celestino Santos-Buelga g, Alfonso Varela-Lopez h, José Luis Quiles h, Maurizio Battino a,d,*

a Dipartimento di Science Cliniche Specialistiche ed Odontostomatologiche (DISCO), Sez. Biochimica, Facoltà di Medicina, Università Politecnica delle Marche, Ancona, Italy
b Escuela de Medicina Veterinaria y Zootecnia, Facultad de Ciencias de la Salud, Universidad de Las Américas (UDLA), Quito, Ecuador
c Research Laboratory, Dental School, University of Sevilla, Sevilla, Spain
d Centre for Nutrition & Health, Universidad Europea del Atlántico, Santander, Spain
e Dipartimento di Scienze Agrarie, Alimentari e Ambientali (D3A), Università Politecnica delle Marche, Ancona, Italy
f Colegio de Administración y Economía (CADE), Universidad San Francisco de Quito (USFQ), Quito, Ecuador
g Grupo de Investigación en Polifenoles (GIP-USAL), Faculty of Pharmacy, University of Salamanca, Campus Miguel de Unamuno, Salamanca, Spain
h Department of Physiology, Institute of Nutrition and Food Technology “José Mataix”, Biomedical Research Center, University of Granada, Spain

* Corresponding authors. DISCO, Facoltà di Medicina, Università Politecnica delle Marche, Via Ranieri 65, 60131 Ancona, Italy. Tel.: +39 071 2204646; fax: +39 071 2204123.
E-mail addresses: f.giampieri@univpm.it (F. Giampieri); m.a.battino@univpm.it (M. Battino).

Abbreviations: AH, artificial honey; MH, Manuka honey; FR, free radical; ROS, reactive oxygen species; RNS, reactive nitrogen species; AAPH, 2,2’-Azobis(2-amidinopropane) dihydrochloride; AMPK, 5’ AMP-activated protein kinase; Nrf2, nuclear factor erythroid 2-related factor; HDFA, primary human dermal fibroblasts; TAC, total antioxidant capacity; CAT, catalase; SOD, superoxide dismutase; OCR, oxygen consumption rate; SRC, spare respiratory capacity; NO, nitric oxide; TBARS, thiobarbituric acid reactive substances

http://dx.doi.org/10.1016/j.jff.2016.05.008
1756-4646/© 2016 Elsevier Ltd. All rights reserved.
1. Introduction

Manuka honey (MH) derived from the _Leptospermum scoparium_ tree has been described to have additional therapeutic advantages in wound health over other honeys, thanks to its high antibacterial capacity (Alvarez-Suarez, Gasparrini, Forbes-Hernández, Mazzoni, & Giampieri, 2014). In addition to the antibacterial capacity of honey, other mechanisms have been associated with their ability to heal wounds, like those related to their physical properties (Molan, 2011). These properties, such as its pH of around 3.2–4.4, favours the topical acidification of wounds and promotes healing by increasing the release of oxygen from haemoglobin (Molan & Rhodes, 2015). Moreover, this pH is less favourable for protease activity, favouring the reduction of the destruction of the matrix needed for tissue repair. Other mechanisms that promote wound healing are related to the immunostimulatory, anti-inflammatory and debriding action of honey (Molan & Rhodes, 2015). MH is also a natural source of a group of polyphenol compounds (Alvarez-Suarez et al., 2014; Chan et al., 2013), which besides their anti-bacterial capacity may synergistically contribute to the healing of wounds.

Clinical reports have demonstrated the capacity of MH in healing chronic wounds in humans and animals. There are many reports of single case studies where MH resulted in healing previously non-healing wounds (Biglari et al., 2012; Bischofberger et al., 2015, 2016; Sell et al., 2012). In fact, MH has been used for treating problematic wounds, like leg ulceration (Gethin & Cowman, 2005); however, the relationship between its healing properties and wound repair mechanism is still unexplored. The purpose of this study was to investigate the potential effects of MH as a potential therapeutic agent against oxidative damage in human skin fibroblasts, which can play an important role in the process of skin wound healing. Special emphasis was made on the possible molecular pathways involved in improving the cellular redox and metabolic state by studying the activation of AMPK/Nrf2 signalling as mainly responsible for the antioxidant response as well as the cascade related to antioxidant defences and mitochondrial functionality.

2. Materials and methods

2.1. Honey samples and reagents

Five samples of pure Manuka honey was obtained from New Zealand Honey LTH, imported in Italy by Efti Srl. An artificial honey (5 samples), reflecting the main components of honey (Copper, Molan, & Harding, 2002), was also included in the study to evaluate the contribution of the predominant sugars to the assayed activities. Primary Human Dermal Fibroblasts (HDFa) isolated from adult skin were purchased from Gibco® Invitrogen cell culture (Life Technologies, Milan, Italy). Media and reagents for cell culturing were purchased from Carlo Erba Reagents (Milan, Italy). All chemicals and solvents were purchased from Sigma-Aldrich Chemical (Milan, Italy). Tali™ Viability Kit-Dead Cell Red, Tali™ Apoptosis assay kit and CellROX® Orange Reagents were purchased from Invitrogen™, Life Technologies. Primary Antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), while goat anti-rabbit IgG peroxidase secondary antibody was purchased from Sigma-Aldrich Chemical (Milan, Italy).

2.2. Analysis of phenolic composition and total antioxidant capacity (TAC) of MH

For phenolic determination, MH was fractionated into Sep-Pak C18 Plus Short SPE Cartridge (Waters S.p.A., Milan, Italy) (Truchado, Ferreres, Borotolotti, Sabatini, & Tomás-Barberán, 2008) and phenolic compounds were identified and quantified by HPLC-DAD-Tandem Mass Spectrometry (MS-MS) using a Hewlett-Packard 1200 chromatograph (Agilent Technologies, Waldbronn, Germany) connected to an API 3200 138 Qtrap (Applied 139 Biosystems, Darmstadt, Germany) mass spectrometer (MS). Then, 280, 330 and 370 nm were selected as preferred wavelengths for the DAD and in the MS, operated in the negative ion mode, spectra were recorded between m/z 100 and m/z 1500. Zero grade air served as the nebulizer gas (30 psi) and as turbo gas (400 °C) for solvent drying (40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). Both quadrupoles were set at unit resolution and EMS and EPI
analyses were also performed. The EMS parameters were: ion spray voltage 4500 V, DP –50 V, EP –6 V, CE –10V and cell exit potential (CXP) –3 V, whereas the EPI settings were: DP –50 V, EP –6 V, CE –25V and CES 0 V.

The TAC of MH was estimated using in parallel the DPPH (Velazquez, Tournier, Mordujovich de Buschiazzo, Saavedra, & Schinella, 2003) and FRAP assays (Benzie & Strain, 1996). In both analyses Trolox was used for the calibration curves and the results were expressed as umoles of Trolox equivalents per 100 g of honey (umol TE/100g of honey). An artificial honey (AH) reflecting the main components of honey was prepared by dissolving 1.5 g of sucrose, 7.5 g of maltose, 40.5 g of fructose and 33.5 g glucose in 17 mL of de-ionized water (Copper et al., 2002). This solution was included in the study to evaluate the contribution of the predominant sugars to the assayed activities.

2.3. Cell culture and treatments

HDFa was cultivated in 25 cm² flasks in EMEM supplemented with 10% foetal bovine serum, 2 mM glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml) in a humidified atmosphere with 5% CO₂ at 37 °C. For cell culture maintenance, medium was changed every 2–3 days and cells were passaged at 80% confluence by trypanosynthesis. For the experiments, cells were used between 8th and 12th passages. Prior to the experiment, fibroblasts were dissociated by trypsinization and then placed in 96-well plates 12 h at a density of 2 × 10⁴ cell/mL. MH was freshly prepared directly diluted in EMEM to achieve the concentration of 0.1%, according to previous cytotoxicity studies, was used as inductor of the oxidative damage in HDFa. AAPH (2-amidinopropane) dihydrochloride (10 mM) was rendered sterile by filtration through Minisart filter of 0.20 µm( P B I n t e r n a t i o n a l , M i l a n, I t a l y ). The stressor 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) (10 mM) was used as inductor of the oxidative damage in HDFa. Cells at 75% confluence were incubated with MH (0.1%) for 24 h and then exposed to AAPH for 0.20 h, and then transferred to the XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica MA, USA) according to the manufacturer’s protocol. Cells were seeded for 16 hours in the XF-24 plate before the experiment and then treated as indicated above. At the end of the treatment the medium was replaced with 450 µL/well of XF-24 running media (supplemented with 25 mM glucose, 2 mM glutamine, 1 mM sodium Pyruvate, without serum) and preincubated at 37 °C for 20 min in the XF Prep Station incubator (Seahorse Bioscience) in the absence of CO₂. The plate was then transferred to the XF-24 Extracellular Flux Analyzer, and after an OCR baseline measurement a profiling of mitochondrial function was performed by sequential injection of four compounds that affect bioenergetics, as follows: 55 µL of oligomycin (2.5 µg/mL) at injection in port A, 61 µL of 2,4-dinitrophenol (2,4-DNP) (1 mM) at injection in port B, and 68 µL of antimycin/rotenone (10 µM/1 µM) at injection in port C. The best concentration of each inhibitor and uncoupler was obtained on the basis of a proper titration curve. Five wells were utilized per condition in any given experiment and data were expressed as pmol of O₂ consumed per minute normalized to 1000 cells (pmol O₂/1000 cells/min). The spare respiratory capacity (SRC) was obtained by calculating the mean of OCR values after injection of 2,4-DNP minus the basal respiration (Brandl & Nicholls, 2011).

2.6. Determination of catalase and superoxide dismutase activity and evaluation of protein and lipid oxidative damage

Catalase activity (CAT) was measured by monitoring H₂O₂ decomposition at 240 nm as a consequence of the catalytic activity of catalase (Aebi, 1984). Superoxide dismutase (SOD) was determined on the basis of inhibition of the formation of NADH–phenazine methosulphate–nitroblue tetrazolium formazan by SOD, measured by spectrophotometry at 540 nm (Kakkar, Das, & Viswanathan, 1984). Protein carbonyl content was determined by the DNPH method (Levine et al., 1990). Lipid peroxidation was measured by the assay of thiobarbituric acid-reactive substances (TBARS) (Ohkawa, Ohishi, & Yagi, 1979), whereas the xylene-orange (FOX2) method was used to determine hydroperoxides (Jiang, Hunt, & Wolff, 1992).

2.7. Evaluation of mitochondria functionality

Oxygen consumption rate (OCR) was measured in real-time using a XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica MA, USA) according to the manufacturer’s protocol. Cells were seeded for 16 hours in the XF-24 plate before the experiment and then treated as indicated above. At the end of the treatment the medium was replaced with 450 µL/well of XF-24 running media (supplemented with 25 mM glucose, 2 mM glutamine, 1 mM sodium Pyruvate, without serum) and preincubated at 37 °C for 20 min in the XF Prep Station incubator (Seahorse Bioscience) in the absence of CO₂. The plate was then transferred to the XF-24 Extracellular Flux Analyzer, and after an OCR baseline measurement a profiling of mitochondrial function was performed by sequential injection of four compounds that affect bioenergetics, as follows: 55 µL of oligomycin (2.5 µg/mL) at injection in port A, 61 µL of 2,4-dinitrophenol (2,4-DNP) (1 mM) at injection in port B, and 68 µL of antimycin/rotenone (10 µM/1 µM) at injection in port C. The best concentration of each inhibitor and uncoupler was obtained on the basis of a proper titration curve. Five wells were utilized per condition in any given experiment and data were expressed as pmol of O₂ consumed per minute normalized to 1000 cells (pmol O₂/1000 cells/min). The spare respiratory capacity (SRC) was obtained by calculating the mean of OCR values after injection of 2,4-DNP minus the basal respiration (Brandl & Nicholls, 2011).

2.8. Measurement of nitric oxide (NO) levels

The production of NO was determined using the Griess reagent (Esposito et al., 2013). Cell culture supernatant (100 µL) was combined with an equal volume of Griess reagent in a 96-well plate, and the nitrite concentration was determined by the absorption of the coloured compound formed by the interaction NO₂/
Griess reagent at 550 nm using the microplate reader (Thermo Scientific Microplate Reader, Multiskan® EX, USA). NaNO₂ (10–100 μM) was used as standard and results were expressed as μM of NaNO₂/10⁶ cells.

2.9. In vitro skin fibroblast migration and proliferation assay

HDFa cells were seeded into 12-well plate and cultured to nearly confluent cell monolayers. Thereafter, a linear wound was generated in the monolayer with a sterile 100 μL plastic pipette tip and free cellular debris was removed by washing once with PBS. After the treatment the medium was removed and cells were washed with PBS, fixed with 4% formaldehyde in PBS at pH 7.4 for 30 min and stained with 20 μM of 10% methylene blue in PBS for 5 min. Excess stain was removed with a series of washing with PBS. The experiments were made in triplicate and five representative images were made for each wound at randomly chosen points. The NIH ImageJ programme was used to analyse the digitized images of the wounds and the mean of the measurements of each treatment group was used to calculate the wound closure rates compared to control. The percentage of wound closures was calculated compared to the control (Esposito et al., 2013).

2.10. Western blot analysis

Whole cellular lysate from HDFa was prepared by gentle shaking with a buffer containing 0.9% NaCl, 20 mM Tris-HCl, pH 7.6, 0.1% triton X-100, 1 mM phenylmethylsulphonylfluoride, 0.01% leupeptin and a cocktail of protease inhibitors (complete cock-
tail). Electrophoresis was carried out in a 10–12% acrylamide SDS/PAGE. AMPK, p-AMPK, Nrf2, Catalase and CuZnSOD antibodies were used to detect proteins by Western blotting. Proteins were electrophoresed, transferred to nitrocellulose membranes, and after blocking were incubated overnight at 4 °C with the respective antibody solution, diluted at 1:500 (v/v). Membranes were then probed with their respective secondary antibody (1:2500, v/v). Immunolabelled proteins were detected by the chemiluminescence method (Immuno Star HRP substrate kit; Bio-Rad Laboratories, Inc.) using the C-DiGit® Chemiluminescent Western Blot Scanner (LI-COR® Biosciences, Carlo Erba Reagents, Milan, Italy) and images were analysed by the Image Studio™ Software version 4.0. The protein was determined by the Bradford method (Bradford, 1976).

2.11. Statistical analysis

Data in figures are given as mean ± SD. Data between different groups were analysed statistically using one-way ANOVA and Turkey’s post hoc test; P < 0.05 was considered as significant and P < 0.01 highly significant.

3. Results

3.1. Phenolic composition and TAC of MH

Up to 16 compounds were identified in MH (Table 1), with leptosin derivatives and methyl syringate as the major compounds, representing approximately 35.5% and 43.87% of the total phenolic content, respectively. The major flavonoids were

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>T_R (min)</th>
<th>[M–H]⁻ (m/z)</th>
<th>MS² (m/z)</th>
<th>Total content (mg/100 g of honey)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unknown</td>
<td>4.6</td>
<td>419,697</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Leptosin derv.</td>
<td>7.1</td>
<td>581</td>
<td>535,323,211,196,179</td>
<td>1.45 ± 0.173</td>
</tr>
<tr>
<td>3</td>
<td>Leptosin</td>
<td>11.5</td>
<td>535</td>
<td>323,245,211,196</td>
<td>0.07 ± 0.011</td>
</tr>
<tr>
<td>4</td>
<td>Phenyllactic acid</td>
<td>15.5</td>
<td>165</td>
<td>147,119</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Methoxyphenyllactic acid</td>
<td>16.5</td>
<td>195</td>
<td>177,163,135</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Dimethoxybenzaldehyde</td>
<td>18.3</td>
<td>165</td>
<td>150,135,122</td>
<td>0.08 ± 0.017</td>
</tr>
<tr>
<td>7</td>
<td>Unknown</td>
<td>19.5</td>
<td>363</td>
<td>225,201,139</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Hexeretin</td>
<td>20.9</td>
<td>301</td>
<td>283,239,233,216,191,149</td>
<td>0.06 ± 0.013</td>
</tr>
<tr>
<td>9</td>
<td>Methyl syringate</td>
<td>21.6</td>
<td>211</td>
<td>196,181,153,137</td>
<td>1.79 ± 0.030</td>
</tr>
<tr>
<td>10</td>
<td>Unknown</td>
<td>23.6</td>
<td>343</td>
<td>325,297,279,219</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Luteolin</td>
<td>24.2</td>
<td>285</td>
<td>257,241,217,199,151,133</td>
<td>0.06 ± 0.008</td>
</tr>
<tr>
<td>12</td>
<td>Quercetin</td>
<td>24.4</td>
<td>301</td>
<td>273,179,151,121</td>
<td>0.09 ± 0.032</td>
</tr>
<tr>
<td>13</td>
<td>Unknown</td>
<td>26.0</td>
<td>672</td>
<td>536,522,496,372</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Kaempferol</td>
<td>28.0</td>
<td>285</td>
<td>229,171</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>15</td>
<td>Pinobanksin</td>
<td>28.3</td>
<td>271</td>
<td>253,243,225,197,161,125</td>
<td>0.28 ± 0.047</td>
</tr>
<tr>
<td>16</td>
<td>Methyllumercetin</td>
<td>28.7</td>
<td>315</td>
<td>301,283,272,164</td>
<td>0.02 ± 0.005</td>
</tr>
<tr>
<td>17</td>
<td>Dimethyllumercetin</td>
<td>33.4</td>
<td>329</td>
<td>314,299,271</td>
<td>0.02 ± 0.003</td>
</tr>
<tr>
<td>18</td>
<td>Chrysir</td>
<td>35.0</td>
<td>253</td>
<td>209,181,143,119</td>
<td>0.02 ± 0.003</td>
</tr>
<tr>
<td>19</td>
<td>Pinocembrin</td>
<td>35.7</td>
<td>255</td>
<td>213,151</td>
<td>0.18 ± 0.035</td>
</tr>
<tr>
<td>20</td>
<td>Galangin</td>
<td>35.9</td>
<td>269</td>
<td>227,213,197,169</td>
<td>0.03 ± 0.008</td>
</tr>
<tr>
<td>Total flavonoid content</td>
<td></td>
<td></td>
<td></td>
<td>0.77 ± 0.021</td>
<td></td>
</tr>
<tr>
<td>Total phenolic content</td>
<td></td>
<td></td>
<td></td>
<td>4.08 ± 0.121</td>
<td></td>
</tr>
</tbody>
</table>

a Values are expressed as means ± standard deviation (SD). Each sample was analysed in triplicate.

b Nondetermined.
3.2. Biomarkers of antioxidant status and oxidative damage

MH protects against apoptosis and cellular death induced by AAPH (Fig. 2A). At 0.1% MH showed no cytotoxic effect in HDFa compared to controls, while AAPH treatment caused significantly lower cell vitality ($P < 0.05$) compared to control. Pre-treatment with MH showed a protective effect on cell viability, with a higher ($P < 0.05$) number of live cells compared to AAPH-stressed cells. In order to evaluate if the protective effect of MH in cell viability is related to changes in apoptotic/death cell numbers, the percentage of live, dead and apoptotic cells after each treatment was analysed. No significant differences were found in apoptotic and dead cell number compared to control after treatment with MH, while after incubation with AAPH the apoptotic and dead cell number was significantly higher ($P < 0.05$) compared to control cells. However, pre-treatment with MH led to significantly lower number of apoptotic and dead cells ($P < 0.01$) compared to AAPH-stressed cells. AH showed no cytotoxic effect in HDFa compared to controls. Pre-treatment with AH did not demonstrate protective effect against oxidative damage caused by AAPH showing a significantly lower cell vitality ($P < 0.05$) when compared to control and MH pre-treated cell (data not shown).

Similar improvements were observed in the markers of antioxidant status and oxidative damage. A significantly higher concentration of intracellular ROS was found after incubation with AAPH compared to control ($P < 0.01$), while pre-treatment with MH showed significantly ($P < 0.01$) lower intracellular ROS levels both compared to control cells and when compared to AAPH-stressed cells ($P < 0.05$) (Fig. 2B). MH was also able to improve CAT and SOD activity and protected proteins and lipids against AAHP-induced stress. A higher activity of SOD ($P < 0.01$) (Fig. 3A) and CAT ($P < 0.05$) (Fig. 3B) was observed after treatment with MH compared to control. Treatment with AAPH affected the activity of both enzymes, causing a lower activity (SOD, $P < 0.05$; CAT, $P < 0.01$), whereas pre-treatment with MH protected against the depression of the enzymatic activity compared with AAPH-stressed cells ($P < 0.01$). MH pre-treatment showed no negative effect on proteins and lipids after 24-hour incubation. Treatment with AAPH caused significantly higher values ($P < 0.01$) of TBARS (Fig. 3C), hydroperoxide levels (Fig. 3D), and protein carbonyl levels (Fig. 3E) compared to control. Pre-treatment with MH significantly protected proteins and lipids against oxidative damage. A significantly lower concentration of TBARS ($P < 0.05$) and hydroperoxides levels ($P < 0.01$) was found when compared with AAPH-stressed cells, whereas similar trends were observed in markers of protein oxidation with a significant decrease ($P < 0.05$) in protein carbonyl levels compared to AAPH-stressed cells. Pre-treatment with AH did not exert protective effect against intracellular ROS production, showing significantly high values of intracellular ROS concentration ($P < 0.01$) after AAPH treatment when compared to MH pre-treated cell (data not shown). Moreover, AH was not able to enhance CAT and SOD activity, or to protect proteins and lipids against AAHP-induced stress ($P < 0.01$), compared to control and MH pre-treated cell (data not shown).

3.3. MH protects mitochondrial functionality against oxidative damage

The protective capacity of MH against the negative effect of AAPH on mitochondrial functionality was assessed by measuring in real time the OCR in cells. Cells were exposed sequentially to each of four modulators of oxidative phosphorylation (OXPHOS), such as oligomycin (an inhibitor of F,FATPase or complex V), 2,4-DNP (uncoupling of the OXPHOS electron transport chain) and antimycin/rotenone (complex I and III inhibitors respectively), and the OCR was determined (Fig. 4). Basal OCR was markedly affected in cells treated with AAPH ($P < 0.01$) compared to control. On the contrary, the OCR was considerably improved ($P < 0.05$) in cells treated with MH before exposition to AAPH compared to AAPH-stressed cells (Fig. 4A). The SRC of cells can be used as an indicator of how close to its bioenergetic limit a cell is operating. Treatment with AAPH led to a significant decrease ($P < 0.01$) of SRC levels compared to control, while MH pre-treatment resulted in a significant improvement ($P < 0.05$) compared with AAPH-stressed cells (Fig. 4B). Pre-treatment with AH had no protective effect on mitochondrial functionality, showing significantly low
OCR and SRC values ($P < 0.05$) after AAPH treatment when compared to MH pre-treated cell (data not shown).

3.4. **MH promotes tissue repair by fibroblast migration and wound closure**

NO is a well-known mediator of normal tissue repair. We therefore tested the ability of MH to induce NO production in HDFa cells. When HDFa cells were treated with AAPH the NO levels were significantly lower ($P < 0.01$) compared to control cells. However, MH led to significantly higher NO levels both compared to control ($P < 0.05$) and to AAPH-stressed cells ($P < 0.01$) (Fig. 5A).

MH was also able to enhance the fibroblast migration and scratch wound closure activity (Fig. 5B) in HDFa. Pre-treatment with MH for 24 hours accelerated scratch wound closure by 39% compared to control ($P < 0.01$). Treatment with AAPH significantly affected ($P < 0.05$) the migration of HDFa and wound closure activity, inducing only 6% compared to control, while pre-treatment with MH was able to counteract the negative effect of AAPH, inducing a scratch wound closure of 32% ($P < 0.01$) compared with AAPH-stressed cells. Again, pre-treatment with AH did not induce NO production and fibroblast migration ($P < 0.05$) after AAPH treatment when compared to MH pre-treated cell (data not shown).

3.5. **The involvement of p-AMPK and Nrf2 signalling in the antioxidant response and tissue repair effects of MH**

To determine the possible involvement of AMPK signal pathways in the tissue repair effect of MH, phosphorylation of AMPK, which is generally linked with AMPK activation, was determined. Treatment with AAPH had no significant effect on the phosphorylation of AMPK. On the contrary, pre-treatment with MH led to a significant ($P < 0.05$) phosphorylation of AMPK when compared to control and to AAPH-stressed cells (Fig. 6).

In order to evaluate the possible relation between the healthy effects of MH and the AMPK and Nrf2 signal pathways, the expression of Nrf2 was also measured by Western blot assay. Treatment with AAPH led to slightly higher Nrf2 protein levels. However, pre-treatment with MH produced significantly ($P < 0.05$) higher Nrf2 protein levels in HDFa cells when compared to control and to AAPH-stressed cells. The expression of two Nrf2 target genes related to antioxidant response enzymes such as...
CAT and CuZnSOD was also measured. Treatment with AAPH caused a lower ($P < 0.01$) expression level for both enzymes compared to control, whereas MH led to a significantly higher transcription of these genes compared to control (SOD, $P < 0.01$; CAT, $P < 0.01$) and to AAPH-stressed cells ($P < 0.01$) (Fig. 6). Pretreatment with AH did not induce the phosphorylation of AMPK, or the stimulation of Nrf2, CAT and CuZnSOD ($P < 0.05$) compared to MH pre-treated cell (data not shown).

4. Discussion

MH has attracted a great deal of attention from researchers for its biological properties, especially for its antimicrobial and additional therapeutic advantages in wound health over other honeys (Biglari et al., 2012; Bischofberger et al., 2015, 2016; Sell et al., 2012). Currently, its antimicrobial effects have been well studied (Alvarez-Suarez et al., 2014; Mannina et al., 2016; Molan & Rhodes, 2015); however, the mechanisms by which MH promotes wound healing are still unknown. Several important pathophysiological processes in wound healing onset and development have been described: oxidative stress, inflammation processes and mitochondrial dysfunction (Martin, 1997; Gurtner, Werner, Barrandon, & Longaker, 2008), processes in which AMPK has been reported to play a master regulatory role (Ruiz, Perez-Villegas, & Carrion, 2005). In this study, we demonstrate for the first time one of the possible mechanisms by which MH exhibits its ability to promote wound healing. This
beneficial property could be due to its capacity to improve antioxidant response through the activation of the signalling pathway AMPK/p-AMPK/Nrf2 and the antioxidant enzymes SOD and CAT. Promotion of the proliferation and migration of fibroblasts might also be involved. It is interesting to note that until now studies on MH have mainly explored its phytochemical composition and antimicrobial activity (Alvarez-Suarez et al., 2014). A few studies on wound healing have focused on proliferation analyses (e.g. in HDF), without investigating the molecular mechanisms related to this capacity (Jull et al., 2015; Ker-Woon, Abd Ghafar, Hui, Mohd Yusof, & Wan Ngah, 2015; Molan & Rhodes, 2015).

In the present study, exposition of HDFa to oxidative stress, exogenously induced by AAPH, caused a significantly lower cell vitality, higher apoptotic and dead cell numbers, intracellular ROS production, TBARS, hydroperoxides and protein carbonyl levels; all of them indicators of oxidative damage. As a consequence of impaired oxidative stress response, the activities of CuZnSOD and CAT, members of the antioxidant protection system regulated by AMPK (Gasparrini et al., 2015), were found to be lower than in the control group. Even though it is true that low levels of antioxidant enzymes may be related to a faster use of these, it also denotes an inability of the cell to properly answer to cell damage.

From the chemical analysis it was evidenced that MH contains a principal phenolic compound family, methyl syringate and derivatives (Kato et al., 2012), which represents more than 75% of the total phenolic content identified, followed by other important phytochemicals such as flavonoids pinobanksin and pinocembrin, representing approximately 36% and 23%, respectively, of the total flavonoid content. Polyphenols have been traditionally known as potent antioxidant molecules, mainly due to their ability to scavenge free radicals (Del Rio et al., 2013). Beyond this, current evidence supports the hypothesis that certain polyphenols have the ability to exert other effects apart from its antioxidant activity (Del Rio et al., 2013). It has been demonstrated that methyl syringate has the scavenging activity of superoxide (Inoue et al., 2005), which has been considered a dangerous free radical for aerobic life. It was also reported that methyl syringate is a selective agonist of transient receptor potential channel, ankyrin 1 (TRPA1) (Son et al., 2012). Studies indicate that TRPA1 acts as a sensor for environmental irritants (Højland, Andersen, Poulsen, Arendt-Nielsen, & Gazerani, 2015), being involved in signal transduction and cellular growth control (Laursen, Bagriantsev, & Gracheva, 2014). Pinobanksin is another major phenolic identified in MH. The protective effect of pinobanksin against oxidative damage was also recently reported. In rats with occlusion-damaged bilat-

Fig. 4 – Oxygen consumption rate (OCR). OCR was monitored using the Seahorse XF-24 Extracellular Flux Analyzer with the sequential injection of oligomycin (2.5 µg/mL), 2,4-DNP (1 mM), and rotenone/antimycin (10 µM/1 µM) at the indicated time point into each well, after baseline rate measurement. (A) Basal OCR levels and (B) spare respiratory capacity in control cells (Control), treatment with Manuka honey (MH), treatment with the stressor AAPH (AAPH) and pre-treatment with Manuka honey and then stressed with AAPH (pret-MH/AAPH). Results are reported as mean ± SD of three experiments. *P < 0.05, **P < 0.01, significant differences compared to control; *P < 0.01, **P < 0.01, significant differences between AAPH and pret-MH/AAPH group.
Fig. 5 – Effect of Manuka honey in promoting tissue repair by nitric oxide production, migration and wound closure in HDFa cells. (A) Production of nitric oxide by HDFa exposed to the different treatments. (B) Effects of Manuka honey in the scratch wound closure in HDFa. (C) Representative images illustrating the migration of HDFa cells into the scratch wound during 24 h exposure. (a) Control, (b) MH, (c) AAPH and (d) pret-MH/AAPH. Results are reported as mean ± SD of three experiments.

* P < 0.05, ** P < 0.01, significant differences compared to control; ### P < 0.01, significant differences between AAPH and pret-MH/AAPH group.
eral common carotid arteries, the supplementation with pinobanksin derivatives (PNC) was able to markedly decrease malondialdehyde levels, enhance superoxide dismutase activity and glutathione levels, and decrease the release of cytochrome c as well as the activities of caspases. PNC also increased Nrf2 and anti-apoptotic bcl-2 protein expression, while Nox1 and pro-apoptotic Bax protein expression was decreased. Thereby, PNC may exert its neuroprotective effects through counteracting oxidative stress (Liu et al., 2015). Another representative flavonoid identified in MH is pinocembrin. Pinocembrin has been reported to exert anti-inflammatory and antioxidant activity. Recent reports indicate that treatment with pinocembrin inhibited LPS-induced inflammatory mediators TNF-α, IL-1β, NO and PGE2 production in BV2 microglial cells. Pinocembrin was able to inhibit LPS-induced iNOS and COX-2 expression, as well as PI3K, Akt phosphorylation, and NF-κB activation, which are required for inflammatory mediator production. Furthermore, treatment of pinocembrin induced nuclear translocation of Nrf2 and expression of HO-1, which conferred protection against MPP(+) induced cytotoxicity (Zhou, Wang, Li, Li, & Geng, 2015). Pinocembrin has been also associated to a neuroprotective effect, even if the mechanism is still unknown. In a recent study it was demonstrated that pinocembrin increased heme oxygenase-1 (HO-1) expression and contribute to shedding some light on the mechanisms whereby pinocembrin inhibits the MPP(+) induced neurotoxicity (Wang et al., 2016). These results might explain, at least in part, the effects found in the present study in relation to cell growth of HDFa after pre-incubation with MH.

A mechanism that could help to explain how polyphenols could exert their antioxidant action in vivo could be the ability of these compounds to up-regulate the activation of the AMPK/Nrf2/ARE (Kelch ECH associating protein 1/NF-E2-related factor 2/antioxidant responsive elements) signalling pathway, and the expression of the antioxidant enzymes like SOD and CAT (Buendia et al., 2016). Nrf2 is a key player in the Nrf2/ARE signalling pathway and is responsible for the expression of the above referred enzymes (Colombo & Moncada, 2009; Lu et al., 2010). In addition, it has been demonstrated that some polyphenols are able to induce, via the Nrf2 pathway, certain phase I and phase II enzymes, which participate in the detoxification of xenobiotics (Han, Shen, & Lou, 2007; Scapagnini et al., 2011). According to the results of the present study, pre-treatment with MH could induce Nrf2 activation throughout AMPK phosphorylation. In this sense, the induction of AMPK phosphorylation by MH could activate Nrf2 accompanied by an increased antioxidant enzyme expression (CuZnSOD and CAT). This, in turn, would lead to an improvement in the protection of HDFa against stress damage, further promoting cell proliferation and therefore wound closure.

Pre-incubation with MH was also associated with a protection on mitochondrial functionality, through considerable improvement in basal OCR and SRC in cells treated with MH.
before exposition to AAPH, compared to AAPH-stressed cells. One of the possible ways through which MH could protect mitochondria could be the possibility of MH polyphenols to cross the cellular membrane and reach the cytosol, exerting their action of radical scavengers intracellularly, as discussed above. Moreover, the capacity to activate several pathways related with cellular antioxidant response, as previously discussed, should also be considered. Currently, particular attention has been paid to the ability of polyphenols to act on a myriad of mitochondrial processes. Some particular polyphenols are now recognized as molecules capable of preserving mitochondrial function by modulating mitochondrial-related pathways, including biogenesis, membrane potential control, electron transport and ATP synthesis, oxidative stress control (i.e., inhibiting/inducing ROS formation/removal enzymes), and ultimately apoptosis (Del Rio et al., 2013; Forbes-Hernandez et al., 2014). Results from the present study may be justified, at least in part, by the above approaches, although further studies should be conducted to determine the direct effect of MH on mitochondrial functionality.

Results presented here reinforce, through scientific knowledge, the beneficial aspects of honey in health. In particular, in the present study, new evidence has been added in support of the effects of MH in wound healing. The results here reported, based on the capacity of MH to improving responsiveness to oxidative damage, as well as stimulation of cell proliferation, could help to understand how MH develops its healing effect on wounds. The proposed mechanism includes the capacity of MH to activate AMPK phosphorylation and subsequent overexpression of the Nrf2/ARE signalling pathway, which is followed by the activation of downstream regulated elements like the antioxidant enzymes SOD and CAT.

Conflict of interest statement

All the authors declare that there is no conflict of interest for any of them.

Acknowledgements

F. Giampieri’s post-doctoral position was supported by a grant from Fondazione Umberto Veronesi, Milano, Italy. The GIP-USAL is financially supported by the Spanish MINECO through Project BFU2012-35228. We are indebted to M. Glebocki for extensive editing of the manuscript.

REFERENCES


