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SHORT COMMUNICATION

Exosome-like vesicles in *Apis mellifera* bee pollen, honey and royal jelly contribute to their antibacterial and pro-regenerative activity

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ABSTRACT

Microvesicles are key players in cellular communication. As glandular secretions present a rich source of active exosomes, we hypothesized that exosome-like vesicles are present in *Apis mellifera* hypopharyngeal gland secretomal products (honey, royal jelly and bee pollen), and participate in their known antibacterial and pro-regenerative effects. We developed an isolation protocol based on serial centrifugation and ultracentrifugation steps and demonstrated the presence of protein-containing exosome-like vesicles in all three bee-derived products. Assessing their antibacterial properties, we found that exosome-like vesicles had bacteriostatic, bactericidal and biofilm-inhibiting effects on *Staphylococcus aureus*. Furthermore, we demonstrated that mesenchymal stem cells (MSCs) internalize bee-derived exosome-like vesicles and that these vesicles influence the migration potential of the MSCs. In an *in vitro* wound-healing assay, honey and royal jelly exosome-like vesicles increased migration of human MSCs, demonstrating their inter-kingdom activity. In summary, we have discovered exosome-like vesicles as a new, active compound in bee pollen, honey and royal jelly.

KEY WORDS: Microvesicle, *Apis mellifera*, Bactericide, Intercellular communication, Inter-kingdom communication

INTRODUCTION

Research into microvesicles is an emerging field following their identification as one of the key factors in intercellular communication. Landmark findings such as the ability to transfer mRNA/microRNA (miRNA) (Valadi et al., 2007) as well their role in the expression of antigens (Raposo, 1996) defined them as the new players in cell-to-cell communication. The recent discovery of exosomes that are not restricted to intra-species interactions but are also capable of inter-kingdom communication has once again revolutionized our understanding of exosomes (reviewed by Schuh et al., 2019). A growing body of literature is describing exosomes isolated from body fluids (including saliva, blood, urine), and animal and plant products as novel sources. For example, snake venom gland-derived exosomes have been demonstrated to cleave regulatory peptides and were shown to be involved in altering blood pressure as well as glucose homeostasis after envenomation (Ogawa et al., 2008).

Honey and royal jelly are produced directly by the hypopharyngeal glands of worker bees, through different mechanisms. To produce honey, adult worker bees convert nectar components by utilizing different enzymes secreted by the hypopharyngeal glands (Kubota et al., 2004; Ohashi et al., 1999). Nurse bees, in contrast, have the ability to synthesize royal jelly *de novo* (Albert et al., 2014) in the hypopharyngeal glands (Haydak, 1970). For the production of bee pollen, pollen is collected from plants, compacted using saliva and nectar, and fermented by bacteria and yeasts in a multi-stage process (Gilliam, 1979).

Bee pollen, honey and royal jelly have demonstrated remarkable antimicrobial and pro-regenerative characteristics. Pre-clinical studies described an improvement of disease scores following royal jelly application in a number of conditions including mucositis, colitis, bone formation and infected ulcers (El-Gayar et al., 2016; Karaca et al., 2010; Özan et al., 2015; Suemaru et al., 2008). Furthermore, honey has been shown to be strongly antibacterial against various bacterial strains (e.g. *Staphylococcus aureus*) (Dustmann, 1979; Maeda et al., 2008) as well as anti-inflammatory (reviewed in Hadagali and Chua, 2014). Bee pollen has been described mostly as a nutraceutical, but has also been shown to exhibit pro-regenerative properties in burn wounds in pre-clinical experiments (Olczyk et al., 2016).

Considering the presence of exosome-like vesicles (ELVs) in gland secretion, we hypothesized that ELVs are present in *Apis mellifera* Linnaeus 1758 hypopharyngeal gland products (bee pollen, honey and royal jelly). Furthermore, we postulate that these ELVs are involved in their known antibacterial and pro-regenerative effects. We describe an adjusted protocol to isolate and characterize ELVs from bee pollen, honey and royal jelly. Furthermore, we unravel their physiological and clinical relevance by investigating their uptake into mammalian cells and characterizing their antibacterial properties in a wound-relevant bacterial strain.

MATERIALS AND METHODS

Isolation of exosomes from bee products

Bee pollen, honey and royal jelly were all obtained in crude, unprocessed form from controlled, organic beekeepers in Chile (Apicola Chile). Honey and royal jelly (Apicola del Alba, Chile) were diluted 1:20 in particle-free phosphate-buffered saline (pf-PBS). Bee pollen was dissolved 1:40 (w/v) in pf-PBS. All samples were centrifuged at 500 ***g***, 1500 ***g*** and 2500 ***g*** for 15 min each and subsequently filtered (0.2 µm). Supernatant was ultra-centrifuged twice at 100,000 ***g*** for 70 min (Thermo Scientific Sorvall). The resulting pellet containing exosomes was resuspended

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in pf-PBS and stored at −80°C until experimentation (scheme depicted in Fig. S1).

Characterization of exosomes

Transmission electron microscopy

To verify vesicle structure, bee pollen, honey and royal jelly isolates were visualized using transmission electron microscopy (TEM). Samples were placed on Formvar/carbon-coated copper meshes (Ted Pella Inc.) for 15 s, counterstained with uranyl acetate for 1 min and subsequently dried for 3 min at 60°C. Vesicles were visualized on a Philips Tecnai 12 with Olympus iTEM software. Representative images of each sample were taken at a magnification of 87,000×.

Nanoparticle tracking analysis

Isolated exosome-like vesicles were analysed for size distribution using nanoparticle tracking analysis (NTA; NS 3000, NanoSight, Malvern, UK). Samples were thawed shortly before measurement, vortexed and diluted 1:100 with pf-PBS. Subsequently, samples were injected manually and measured at camera level 8 in a temperature-controlled environment (25°C) for 60 s per sample.

Exosomal protein quantification

The bicinchoninic acid assay (BCA; Pierce, Thermo Fisher Scientific) was used for protein quantification. Isolated exosome-like vesicles were lysed in Tris/SDS/deoxycholate buffer for 15 min. Subsequently, samples were incubated at 60°C for 30 min and measured at 562 nm using a microplate reader (Infinite, Tecan, Männedorf, Switzerland).

Antibacterial and biofilm inhibition assays

Staphylococcus aureus was chosen to determine the potential antibacterial and antibiofilm effect of bee product-derived ELVs, as it is a prevalent bacterial strain involved in wound infections (Bowler et al., 2001). Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of ELVs were determined utilizing *S. aureus* ATCC 25923 (Treangen et al., 2014), whereas minimum biofilm inhibition concentration (MBIC) was assessed on *S. aureus* ATCC 29213, a known biofilm-forming strain (Mottola et al., 2016). Both *S. aureus* strains were maintained on tryptic soy broth agar plates (TSB; Becton Dickinson GmbH), and grown for 24 h at 37°C. For experiments, all bacteria were prepared according to guidelines from the Clinical and Laboratory Standards Institute (2014).

Briefly, MIC and MBC were determined as follows. Viable *S. aureus* ATCC 25923 cells were inoculated into 96-well plates at a concentration of 5×10^4 colony forming units (CFU) per well. ELVs (ratios: 0.01:1, 0.05:1, 0.1:1, 1:1, 1:10 and 1:100 vesicles per CFU) were prepared in 10 µl PBS and added to the wells. Chlorhexidine (0.0125%, 10 µl) and ampicillin (300 mmol l^{-1}, 10 µl) served as negative growth controls, 10 µl PBS as a vehicle control, and *S. aureus* in TSB as a positive growth control. Plates were incubated for 24 h at 37°C and 5% CO_2. MIC was determined as the lowest concentration of ELVs that inhibited growth in the wells. Addition of 0.016% resazurin (Sigma-Aldrich) to the wells, with a further 1 h incubation at 37°C, was utilized to confirm bacterial growth inhibition. Aliquots (2.5 µl) from growth-negative wells were incubated on TSB agar plates and checked for bacterial growth after 24 h, and MBC was determined as the lowest concentration of ELVs that completely eradicated viable bacteria growth.

For evaluation of biofilm formation, *S. aureus* ATCC 29213 were grown in TSB (with 1% glucose) in 96-well microplates for 24 h at 37°C and 5% CO_2, with ELV ratios of 0.05:1, 0.1:1, 1:1, 1:10 and 1:100 vesicles per CFU. MBIC was determined as the lowest concentration of ELVs that inhibited biofilm formation in the well. For quantitative evaluation, biofilms were dried, stained with a Crystal Violet solution (0.1% in dH_2O) for 1 h, and washed with dH_2O. Subsequently, 100 µl of 95% ethanol was used to liberate the Crystal Violet from the biofilm, and biomass was determined by absorption at 590 nm (Sunrise, Tecan).

As the antibacterial effect of honey has been reported by several groups and attributed to compounds such as methylglyoxal (reviewed in Kwakman and Zaat, 2012), the role of exosomes within the crude product was assessed. Honey and royal jelly were diluted 1:2 in pf-PBS and bee pollen was dissolved in pf-PBS at a concentration of 1 g 5 ml^{-1}. To establish an exosome-depleted fraction, all three bee products were centrifuged for 15 min at 500 ***g*** and compared with exosome-containing bee pollen, honey and royal jelly at 20%, 10%, 5% and 1% v/v concentrations as previously reported by Almasaudi et al. (2017) MIC, MBC and MBIC were assessed as described above.

Exosome internalization assay

Internalization of royal jelly exosomes into mammalian cells was assessed with carboxyfluorescein-succinimidylester (CFSE)-stained exosomes using confocal microscopy for qualitative analysis and flow cytometry for quantitative analysis. Bee pollen, honey and royal jelly exosomes were incubated with 4 µmol l^{-1} CFSE (Cell Trace CFSE, Thermo Fisher Scientific) for 90 min at 37°C, and subsequently washed twice with pf-PBS followed by ultracentrifugation. For confocal microscopy, human mesenchymal stem cells (MSCs), isolated from the umbilical cord and cultivated as previously described (Cuenca et al., 2018), were seeded onto glass coverslips (5000 cm^{-2}) and left to adhere for 24 h, and subsequently CFSE-stained exosomes (2.5×10^4 per 1000 cells) of all three groups were added. After incubation for 16 h, cells were washed 3× with pf-PBS to remove residual exosomes and fixed with 4% formaldehyde (Sigma-Aldrich) for 30 min. Nuclei were stained with Hoechst dye (Sigma-Aldrich) and prior to mounting, samples were washed 5× with PBS. Images were taken on a Leica 6000 confocal microscope (Leica; software LAS X version 3.3.0.16799).

For flow cytometry, MSCs were seeded into 6-well plates (5000 cm^{-2}), left to adhere for 24 h and incubated for 16 h with CFSE-stained exosomes (2.5×10^4 per 1000 cells) of all three groups. Unstained MSCs and CFSE-stained MSCs (5 µmol l^{-1} CFSE in PBS for 8 min) served as controls. Subsequently, cells were detached using trypsin-EDTA, washed thoroughly with PBS to remove excess exosomes, and measured in a flow cytometer (BD FACS Canto II, 10,000 events). For data analysis, FlowJo software (TreeStar, version 8.8.6) was used.

Cellular migration assay

The effect of exosomes on cellular migration was evaluated with a scratch assay. Human MSCs were seeded in a 12-well plate (35,000 cm^{-2}) and left to adhere overnight. Subsequently, a scratch was inflicted using a pipette tip and the cell layer was washed twice with PBS to remove residual cells as well as fetal bovine serum (FBS). FBS-free medium, containing 10^7 bee pollen, honey and royal jelly exosomes – or the respective volume of PBS – was added. Scratch closure was assessed after 4, 8 and 24 h, and compared with that at 0 h.

Statistical analysis

All data in this study are shown as means±s.d. and were tested for normal distribution. Statistical analysis was performed using

one-way ANOVA followed by Tukey's range test for significant differences between means. Significance was considered at $P<0.05$ (see figure legends for specific values). For statistical calculations, GraphPad Prism 5 for Mac OS X, version 5.0b (GraphPad Software, Inc.), was used. *N* values were determined from independent experiments and independent isolations of ELVs, not as technical replicates.

RESULTS AND DISCUSSION

Evidence of ELVs in bee pollen, honey and royal jelly

The presence of vesicles in the isolated fraction of bee pollen, honey and royal jelly was verified using TEM and NTA as shown in Fig. 1A,B. All three bee products contained vesicles in the size range of exosomes (<150 nm). The mean (±s.d.) particle sizes were 171.1±13.9 nm for bee pollen, 186.5±14.2 nm for honey and 121.7 ±15.1 nm for royal jelly. The highest intra-specimen purity of exosome-like particles was found for royal jelly, with its mean particle size not significantly varying from its particle mode (112.2±4.9 nm), followed by bee pollen (135.2±4.4 nm) and honey (148.05±10.9 nm) (Fig. 1C). Particle count normalized to crude product (particles per gram) revealed higher amounts of particles in bee pollen ($7.43\times10^9\pm4.5\times10^9$) and honey ($4.9\times10^9\pm3.5\times10^9$) compared with royal jelly ($3.9\times10^9\pm3.4\times10^8$). There was no difference in protein concentration between bee pollen and honey, but significantly less protein in royal jelly. However, a larger amount of particles in the exosome range was detected in royal jelly, in both relative and absolute terms (royal jelly $3.5\times10^9\pm3.4\times10^8$>bee pollen $3.37\times10^9\pm1.6\times10^9$>honey $1.9\times10^9\pm1.1\times10^9$). The relative amount of ELVs compared with total vesicles (i.e. purity) was significantly higher in royal jelly (88.25%) than in bee pollen (47.03%) and honey (42.38%).

Antibacterial effects of bee-derived ELVs

To analyse the potential antibacterial and biofilm-inhibiting effect of bee-derived ELVs, we determined the MIC, MBC and MBIC for *S. aureus* (Fig. 2). The MIC for royal jelly was found to be at a ratio of 0.05:1 vesicles to CFU, while honey and bee pollen had MIC values of 1:1 vesicles to CFU. Resazurin-based colorimetric

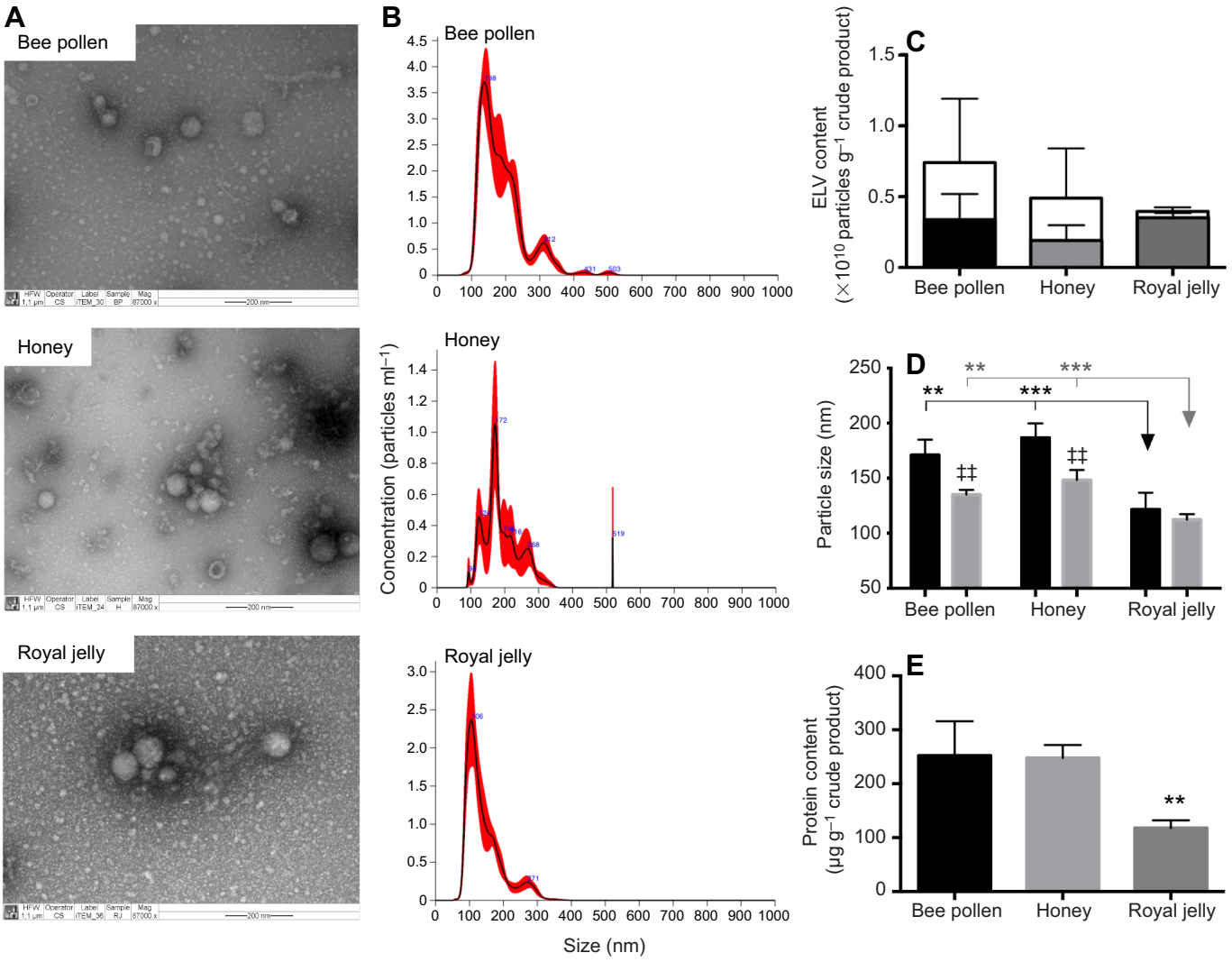


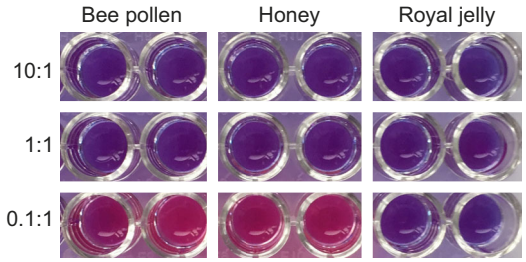
Fig. 1. Bee pollen, honey and royal jelly display vesicles in the range of exosomes (<150 nm). (A) Transmission electron micrographs of exosome-like vesicles (ELVs) derived from bee pollen, honey and royal jelly. (B) Representative histograms of particle distribution from nanoparticle tracking analysis (NTA). (C) NTA analysis of mean particle count (white) versus exosome count (<150 nm; shaded) per 1 g crude product. (D) Mean (black) and mode (grey) of respective vesicle size from NTA analysis. (E) Protein content normalized to 1 g crude product, measured with the BCA assay. *n*=4, means+s.d. **$P<0.01$, ***$P<0.001$; ‡‡$P<0.01$ between mean and mode of the same bee product.

Journal of Experimental Biology (2019) 222, jeb208702. doi:10.1242/jeb.208702

A

S.aureus ATCC 25923

	MIC	MBC
Bee pollen	1:1	1:1
Honey	0.1:1	1:1
Royal jelly	0.05:1	0.1:1



B

S.aureus ATCC 25923

	MBIC
Bee pollen	10:1
Honey	10:1
Royal jelly	1:1

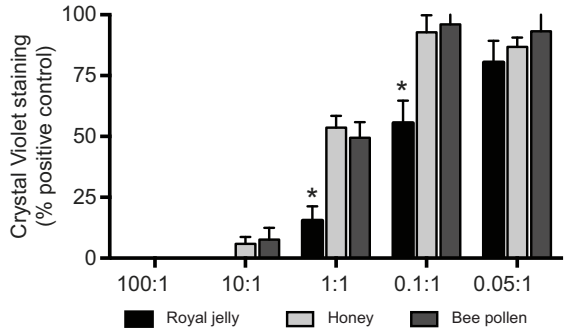


Fig. 2. Bee pollen-, honey- and royal jelly-derived ELVs display antibacterial and biofilm-inhibiting properties. (A) The table shows the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of bee pollen-, honey- and royal jelly-derived ELVs on *S. aureus* ATCC 25923, as the ratio of ELVs to bacteria (n=4). Representative images of resazurin metabolic activity staining in a 96-well plate, at concentrations of 10:1, 1:1 and 0.1:1, are shown below. (B) The table shows minimum biofilm inhibition concentration (MBIC) of bee pollen-, honey- and royal jelly-derived ELVs on *S. aureus* ATCC 29213, as the ratio of bacteria to exosomes after visual assessment. The bar graph below gives numeric results of Crystal Violet biofilm staining as a percentage of positive control (n=4). Means+s.d. *P<0.05.

analysis confirmed visual MIC findings. Regarding the MBC, all three ELVs demonstrated bactericidal effects at a ratio of 0.1:1 vesicles to CFU.

Furthermore, all tested bee-derived ELVs demonstrated biofilm-inhibitory capacity, with MBIC values at ratios of 1:1 vesicles to CFU for royal jelly, and at a ratio of 10:1 vesicles to CFU for both honey and bee pollen. However, a 50% biofilm inhibition was observed at ratios of 0.1:1 for royal jelly and 1:1 for both honey and bee pollen. Finally, a significantly increased biofilm-inhibition capacity was observed for royal jelly compared with honey and bee pollen at ratios of 0.1:1 and 1:1 vesicles per viable bacterial cell.

Assessing the role of ELVs within the antibacterial properties of the crude products, we found that exosome-depleted bee pollen and royal jelly displayed inhibitory and bactericidal effects at 5% (v/v), while exosome-rich bee pollen and royal jelly inhibited bacterial growth at 1%. No difference was found for MIC/MBC for exosome-depleted honey (both 1%). Concentrations of 20% and 10% led to the strongest inhibition of biofilm formation. However, at 10%, significant differences were observed for the exosome-depleted compound. Interestingly, in contrast to its isolated vesicles, crude royal jelly displayed a significantly lower biofilm-inhibitory capacity compared with bee pollen and honey (Fig. S2).

Bee-derived ELVs are internalized by human MSCs

Confocal imaging of human MSCs incubated with CFSE-stained vesicles revealed a localization of vesicles from all three sources in the plasmatic membrane of human MSCs. Representative images are shown in Fig. 3A. Quantitative analysis using flow cytometry confirmed the uptake into MSCs (Fig. 3Bi). Furthermore, it revealed that significantly more royal jelly-derived vesicles were internalized compared with those from honey and bee pollen (royal jelly 31.33±5.2% >> honey 20.31±5.8%>bee pollen 18.32±4.1% cells positive for CFSE) (Fig. 3Bii).

Bee-derived ELVs promote cell migration

To understand the biological significance of the uptake of bee-derived exosomes by mammalian cells, we assessed their effect in a cell migration assay (Fig. 3C,D). Bee pollen-derived ELVs had no effect on the migration of MSCs (scratch closure control: 67.2 ±6.7%; bee pollen: 38.4±4.0%). Interestingly, only 2% more honey-derived ELVs were internalized by MSCs, but they significantly increased cellular migration, resulting in scratch closure of 84.7±4.3% after 24 h. Royal jelly-derived ELVs displayed the most pronounced pro-migratory effect, leading to 96.1±2.1% scratch closure.

Discussion and conclusions

To our knowledge, this is the first study identifying ELVs in bee hypopharyngeal gland-derived products. Interestingly, Van Vaerenbergh et al. (2014) suggested the presence of exosomal proteins in venom from *A. mellifera*; however, they did not further investigate the exosomes themselves. All three examined isolates (bee pollen, honey and royal jelly) revealed the presence of ELVs, which was visually verified by TEM and confirmed by NTA as well as the presence of proteins in the vesicles (Fig. 1). Interestingly, differences could be observed amongst particle count profiles. While honey and bee pollen displayed a wider range of particle sizes, royal jelly presented a homogeneous particle profile (Fig. 1A, B), which can be explained by high amounts of plant particles in the crude products. Honey has been shown to contain plant particles as well as plant miRNAs (Gismondi et al., 2017), and bee pollen is fermented from pollen in the presence of saliva and nectar (Gilliam, 1979). Royal jelly, in contrast, is produced *de novo* by honeybee hypopharyngeal glands in the absence of plants. This may explain

Journal of Experimental Biology (2019) 222, jeb208702. doi:10.1242/jeb.208702

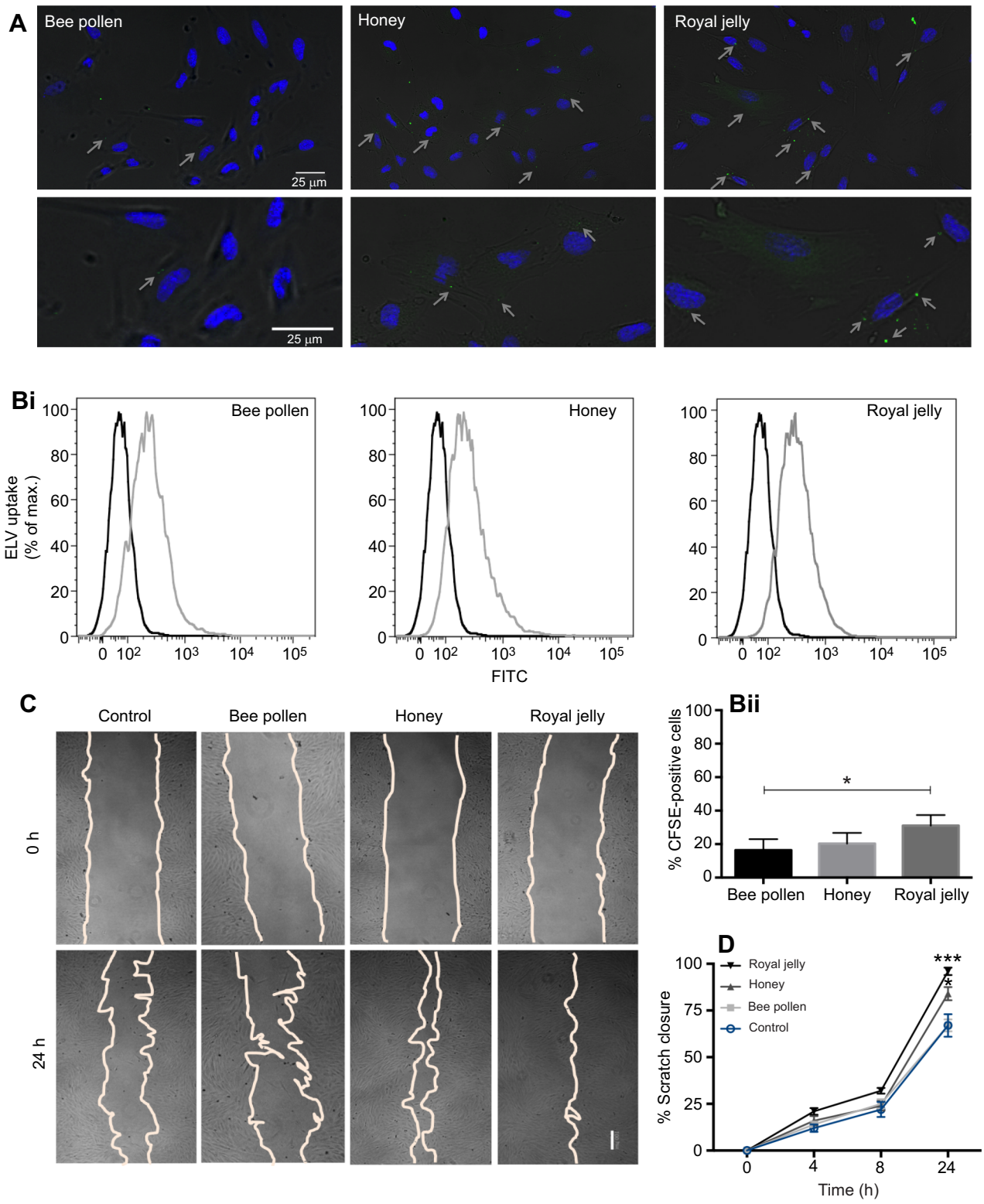


Fig. 3. Bee pollen-, honey- and royal jelly-derived ELVs are internalized into human mesenchymal stem cells (MSCs) and promote migration. (A) Confocal imaging of MSCs after 16 h incubation with carboxyfluorescein-succinimidylester (CFSE)-stained ELVs (arrows); nuclei were stained with Hoechst. (B) Flow cytometric quantification of ELV uptake into human MSCs after 16 h incubation with CFSE-stained ELVs (i) and percentage of CFSE-positive cells (ii). (C) Representative phase contrast micrographs at time point 0 and 24 h. Scale bar: 10 µm. (D) Quantitative analysis of scratch closure (0, 4, 8 and 24 h) of MSCs incubated with ELVs of bee pollen, honey and royal jelly origin (n=4). Means+s.d. *P<0.05, ***P<0.001.

the increased homogeneity and stronger pro-migratory and antibacterial effects observed for royal jelly-derived ELVs throughout this study.

Regarding functional properties, this study is the first to report the antibacterial and biofilm-inhibitory properties of bee-derived ELVs (Fig. 2). For these experiments, we proposed a novel approach,

which utilizes ratios of exosomes (vesicle number) to bacterial CFU in order to determine their effect on *S. aureus* viability. Overall, this approach allowed effective and reproducible handling of exosome concentrations throughout all antibacterial and anti-biofilm experiments. Our results demonstrate that bee-derived ELVs exert *in vitro* antibacterial and biofilm-inhibitory capabilities on *S. aureus* strains, which suggests that these vesicles could help prevent and/or treat wound-derived infections during the healing process. Amongst the three studied products, royal jelly demonstrated increased inhibitory capacity for both the growth of bacteria in solution (MIC) and biofilm formation on surfaces, compared with honey and bee pollen. Interestingly, royal jelly ratios of less than 1 vesicle per viable bacterium demonstrated bacteriostatic and bactericidal effects on *S. aureus* cells and were able to reduce biofilm formation by about 50%.

Previous literature reports antibacterial effects of crude honey and royal jelly on a number of strains (McLoone et al., 2016). Almasaudi et al. (2017) observed an antibacterial effect of different types of honey on a number of methicillin-resistant and methicillin-sensitive *S. aureus* strains (including *S. aureus* ATCC 29213), at concentrations as low as 10% (v/v). Furthermore, Voidarou et al. (2011) and Matzen et al. (2018) evaluated regional varieties of honey in Greece and Denmark, respectively, and found similar antibacterial properties regarding *S. aureus*. Regarding the antibacterial effect of royal jelly, Garcia et al. (2013) observed that raw royal jelly as well as a derived ethyl ether extract inhibited the growth of several bacterial strains including *S. aureus*, at concentrations as low as 30% (w/w) in agar diffusion tests. Similar results were previously reported by Eshraghi and Seifollahi (2003), who observed growth inhibition of *S. aureus* with both pure royal jelly and two different royal jelly fractions. Several compounds and factors have been described as potential mechanisms behind the antimicrobial activity of honey and royal jelly, such as methylglyoxal, royal jelly proteins and oligosaccharides and jelleins (reviewed in Brudzynski et al., 2011; Fratini et al., 2016; Israili, 2014). We demonstrate that depleting bee pollen, honey and royal jelly from exosomes leads to a decrease in their antibacterial and biofilm-inhibitory activity (with the exception of honey for MIC/MCB; Fig. S2), proposing that purified ELVs – in the absence of other factors – contributes to the antibacterial effect of these bee-derived products.

In the present study, bee product-derived ELVs were also found to inhibit biofilm formation in our *in vitro* model (Fig. 2B). Biofilms are surface-adhered bacterial communities that are responsible for many diseases including impaired wound healing (Omar et al., 2017; Roy et al., 2019). Therefore, the antibiofilm effect of bee-derived ELVs on *S. aureus*, paired with their pro-migratory activity, could potentially be used in treatments to improve wound healing in clinics. Some previous studies have reported antibiofilm activity of honey and honey-derived defensin-1 on wound pathogens and species (Majtan et al., 2014; Sojka et al., 2016); however, our results are the first to demonstrate that ELVs possess a biofilm-inhibitory effect on *S. aureus*, across all three bee-derived compounds tested.

One of the key findings of this study is the uptake of bee-derived vesicles into mammalian cells (Fig. 3A,B), combined with their ability to subsequently influence mammalian cell behaviour (Fig. 3C). Exosomes from several sources such as plants and bacteria have been found to participate in inter-kingdom communication (reviewed in Schuh et al., 2019), and our results are the first to demonstrate that bee-derived vesicles are internalized by MSCs. As with purity and homogeneity, we observed a significantly higher uptake of royal jelly exosome-like particles compared with honey and bee pollen ELVs. Given the size differences as well as heterogeneity of the crude products, it can be speculated that different uptake mechanisms (e.g. micropinocytosis, membrane fusion or clathrin-dependent uptake) influence the uptake efficacy (Mathieu et al., 2019).

Analysing the significance of increased MSC migration after uptake of bee-derived ELVs, some considerations have to be acknowledged. Honey and royal jelly have both been demonstrated to exert pro-regenerative properties in cutaneous wound healing (Efem, 1988; El-Gayar et al., 2016); however, the underlying mechanisms have not been fully elucidated yet. ELVs from these two sources significantly increased migration of MSCs, while bee pollen did not change the migratory behaviour. In mammalian wound healing models, it has been shown that one of the crucial steps is migration of stem cells to the wound site to assist in immunomodulation, recruitment of fibroblasts and regeneration (Cerqueira et al., 2016; Pelizzo et al., 2015). Furthermore, an increased migratory potential of MSCs has been associated with improved wound healing (Lau et al., 2009). The effects of honey- and royal jelly-derived ELVs on wound healing *in vivo* have yet to be determined; however, the significant promotion of MSC migration by these vesicles is promising.

In summary, we have discovered ELVs as a new, active compound in bee pollen, honey and royal jelly. These ELVs participate in antibacterial properties and are internalized by human MSCs, influencing their migratory behaviour. Honeybee derived-products such as honey and royal jelly have been used since ancient times in various cultures over the globe for their antimicrobial and pro-regenerative properties (summarized in Ahmed et al., 2003; Moore, 1976). However, with the onset of modern medicine, and the regulation and standardization of medicinal products, their use has decreased significantly. Utilizing bee product-derived exosomes appears to be a promising option to standardize medical applications of bee products and, on the basis of scientific evidence, bring traditional medicine into the 21st century. Nevertheless, further studies are needed to assess exosome cargo and functional differences between bee pollen-, honey- and royal jelly-derived ELVs, to fully understand their role in the biological effect of these substances.

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Competing interests
M.K. is the CSO of Cells for Cells and Consorcio Regenero. G.Z. received stipends from Consorcio Regenero.

Author contributions
Conceptualization: C.M.A.P.S., M.K.; Methodology: C.M.A.P.S., S.A., G.Z.; Validation: C.M.A.P.S., S.A.; Formal analysis: C.M.A.P.S., G.Z.; Investigation: C.M.A.P.S., M.K.; Data curation: C.M.A.P.S., G.Z.; Writing - original draft: C.M.A.P.S., S.A., M.K.; Writing - review & editing: S.A.; Project administration: C.M.A.P.S.; Funding acquisition: C.M.A.P.S.

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