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The preliminary effect of Manuka honey on cancer stem-like cells from colonspheres

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Abstract

Honey has always been used not only as a food/sweetener but also as a medicine since ancient times. The quality and composition depend on many factors, including the botanical origin, the environmental, processing and storage conditions[1]. The nutritional characteristics and the preventive/therapeutic effect of honey are due to its composition: it contains over 180 different types of compounds including water, carbohydrates, enzymes, amino acids, minerals, vitamins and different phytochemicals[2] . Among the different effects on health, those most studied in the scientific literature are its antibacterial activity[3] and the antioxidant capacity[4]. In recent years the potential anticancer effect of honey, in several tumor cell lines, has also been a reason of study[5]. Among different types of honey, Manuka, has shown a high anticancer effect, especially in colon cancer cells (LoVo and HCT-116)[6,7] while it is little known on its effect in cancer stem cells' (CSCs; a rare population of cells within the tumor mass that seem to be responsible for the tumor onset) chemoresistance and the presence of relapse [8]. Therefore, the effect of Manuka honey on CSCs-like was evaluated. In general, CSCs-like were enriched from the monolayer population of HCT-116 through the in vitro sphere forming assay[9]. This honey was able to modify the morphological parameters of the spheroids, reducing the size and volume of the entire culture. The treatment of CSCs-like enriched colonospheres with Manuka honey also led to an intracellular accumulation of ROS and induction of apoptosis. Furthermore, through real-time PCR, down-regulation of ABCG2 gene expression (one of the efflux pumps closely associated with the chemoresistance phenotype) was observed.

Material and Methods

➢ *Honey sample and treatment preparation*

Manuka honey Nèctar Plus® was weighted and dissolved into the complete medium. Before being placed in the culture containing the formed spheroids it was properly filtered with MF-Millipore™ Membrane Filter, 0.45 µm pore size.

➢ *In vitro sphere forming assay for the enrichment of CSCs-like population (Fig.1)*

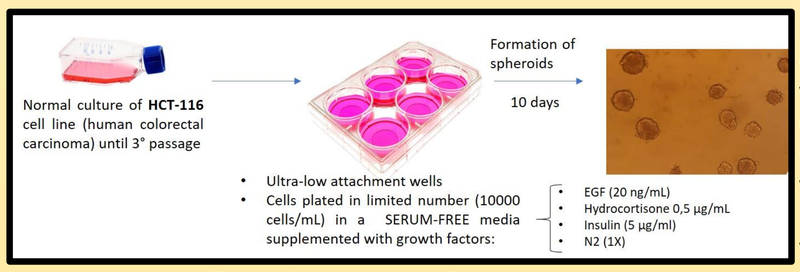


Figure 1. In vitro sphere forming assay

From 2D culture of HCT-116 colon cancer cell line at 3° passage, cells were collected and seeded at low density (10000 cells/mL) in a serum-free medium supplemented with different growth factors. After 10 days the spheroids enriched with CSCs-like are formed (Fig.1).

➢ *Multi parametric spheroid-toxicity assay (Image acquisition and 3D image analysis)*

After the treatment of spheroids with MH (0, 50, 75 mg/mL for 48 h), brightfield images of both the entire culture of the spheroids and the single spheroids subjected to different treatments were captured with the microscope. The images obtained were processed using two open-source software (ReViSP and AnaSP). The 3D volume of spheroids, the sphericity, solidity and the length of the minor diameter were evaluated; this parameters are considered valid for evaluating cytotoxicity in spheroids.

➢ *Determination of intracellular ROS levels and apoptosis assay*

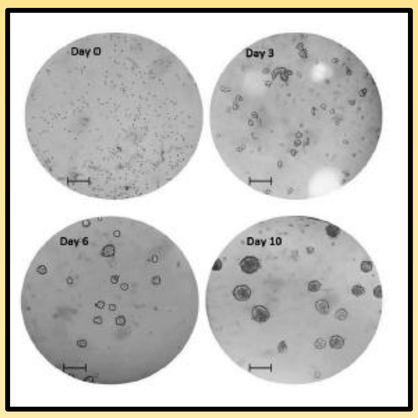
After the treatment of spheroids with MH (0, 50, 75 mg/mL for 48 h), Intracellular ROS accumulation in CSCs-like from spheroids was determined with CellROX® Orange asay kit; Live, apoptotic and dead cells were established by staining cells with Annexin Alexa Fluor® 488 and PI. Both assays were processed with Tali™ Cytometer.

➢ *RNA isolation and quantitative real-time PCR analysis for the evaluation of ABCG2 gene expression*

The spheroids were treated with different concentrations of MH (0, 50, 75 mg/mL) for 48 h. Total RNA was isolated from CS-like cells forming the spheroids and HCT-116 grown in monolayer conditions without treatment, RNA purity and concentration were checked using a microplate spectrophotometer system. Reverse transcription was used to obtain 100 ng of cDNA. GAPDH was used for normalizing the quantitative data, expressed as a relative mRNA level compared to the control. The $2^{-\Delta\Delta Ct}$ method was used for calculating the fold-change value.

Results

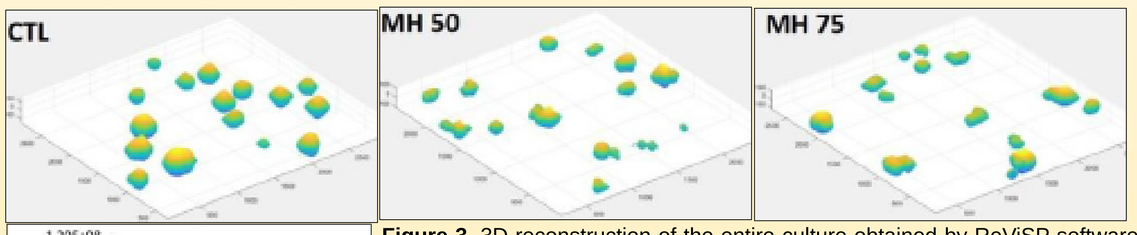
➢ *Formation of colonospheres enriched with CSCs-like*



The *in vitro* sphere forming assay in serum-free medium with the presence of appropriate growth factors permit to enrich the population of CSCs-like cells that grow like spheroids (Fig. 2); this method, in addition to studying the effect of a treatment on this rare cell population, also offers the opportunity to study a 3D experimental model that better mimics the tumour environment compared to a normal monolayer 2D culture.

Figure 2. Enrichment of colorectal CSCs-like by *in vitro* sphere forming assay from day 0 to 10. Scale bar=100 µm.

➢ *MH reduces the volume of the entire culture and affects morphological parameters of the single spheroid*



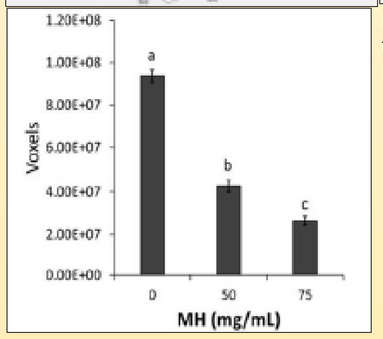


Figure 3. 3D reconstruction of the entire culture obtained by ReViSP software and the subsequent evaluation of its volume was acquired by software and expressed in voxels..

The 3D reconstruction of the entire culture was obtained by ReViSP software and the subsequent evaluation of its volume was acquired and expressed in voxels (Fig.3). In the images representing the 3D reconstruction. The reduction in volume is then confirmed by the histogram: there is a statistically significant decrease ($p<0.05$) in the volume of the entire culture when the spheroids were treated with the MH at the lowest concentration, with an even more accentuated reduction when a concentration of 75 mg/mL is used.

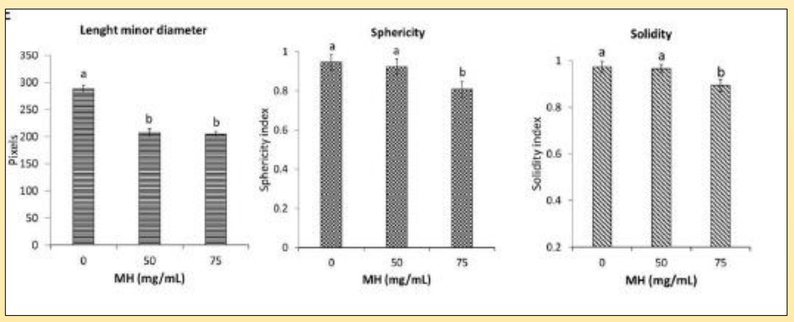


Figure 4. Histograms of the values of some morphological parameters (estimated with AnaSP software and expressed in pixels) closely related to the effect of treatment with drugs: length of minor diameter, sphericity and solidity.

From the histograms (Fig. 4) it is possible to notice that the treatment of single spheroids with MH significantly ($p<0.05$) decrease the lenght of minor diameter; at the same time, the sphericity and solidity of spheroids are significantly reduced only when they are treated with the high concentration of MH.

➢ *MH increases intracellular accumulation of ROS and induces apoptosis in colorectal CSCs-like from spheroids*

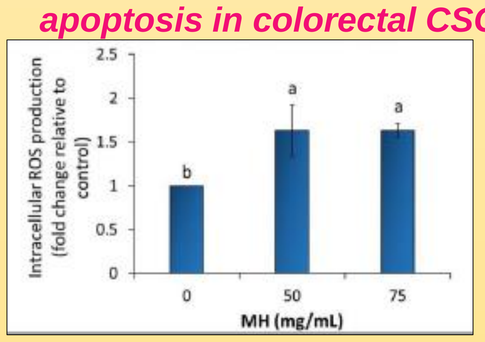


Figure 5. Histograms of the intracellular ROS production (fold change relative to control)

The treatment of spheroids with MH (50, 75 mg/mL for 48 h) significantly *(p<0.05)* increased intracellular ROS production (Fig. 5) and apoptotic cells (Fig. 6), targeting one of the most important feature of CSCs: the survival ability.

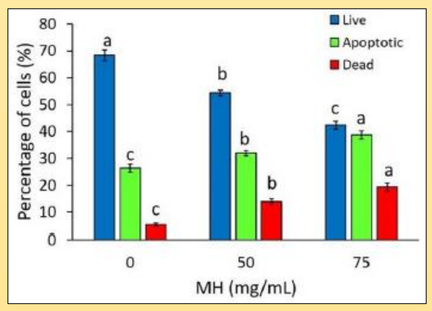


Figure 6. Histograms of the percentage of live (blu), apoptotic (green) and dead (red) cells.

➢ *MH in higher concentration decreased mRNA of ABC-transporters in colorectal CSCs-like from spheroids*

It was observed that in colorectal cancer an over-expression of ABCG2 is associated with a higher resistance to different chemotherapy drugs. In this study the levels of ABCG2 gene expression in HCT-116 cells grown in monolayer and in CSCs-like from spheroids treated with MH (50 and 75 mg/mL) were evaluated and compared. As shown in Fig. 7, mRNA levels of ABCG2 in cells grown in a monolayer culture were significantly lower *(p<0.05)* than in CSCs-like from spheroids. Treatment of colonospheres with the lowest concentration of MH did not decrease the gene expression of this efflux pump which instead increased; conversely, a significant decrease occurred with the use of the highest concentration of MH which, however, did not reach the lowest levels of that found in 2D cultivated cells.

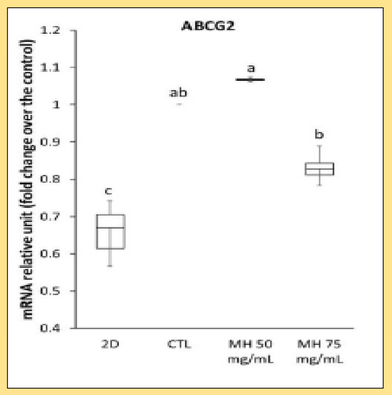


Figure 7. Manuka honey reduces mRNA expression of ABCG2 in colorectal CSCs-like from spheroids.

Conclusion

The results obtained confirm our initial hypothesis: MH, in particular the highest concentration (75 mg/mL), was able to suppress some fundamental features of colon CSCs-like. MH in fact decreased the volume of the entire culture of spheroids enriched with CSCs-like, also modifying some fundamental morphological parameters, as well as inducing apoptosis, probably due to the increased accumulation of intracellular ROS. It is interesting to note that the higher concentrations of MH reduced gene expression levels of the ABCG2 pump, one of the factors responsible for chemoresistance in colorectal cancer, because it increased the extrusion of chemotherapy drugs outside the cell

References

(1) DOI: 10.1016/j.lwt.2017.08.079; **(2)** DOI: 10.3390/molecules23092322; **(3)** DOI: 10.1155/2019/2464507; **(4)** DOI: 10.1016/j.jff.2016.05.008; **(5)** DOI: 10.1016/j.clnu.2018.12.019; **(6)** DOI: 10.1039/c8fo00164b; **(7)** DOI: 10.1039/c8fo00165k; **(8)** DOI: 10.1016/j.phrs.2018.08.006; **(9)** DOI: 10.18632/oncotarget.6261.