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Royal jelly suppresses invasive potential of colorectal cancer cells by attenuating Vimentin and Snail †

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Abstract: Royal jelly (RJ), as an exclusive bee product, showed beneficial effects on various human pathological conditions, including cancer. Therefore, we examined the effects of RJ on suppression of invasiveness of SW-480 cells and protein expression of Vimentin and Snail, markers of cancer cell invasive properties. This natural treatment suppressed agressive behavior of tested cells by inhibiting expression of transcription factor Snail, thus consequently expression of cytoskeletal protein Vimentin with key role in supporting invasive potential. In conclusion, our report indicate the possible molecular mechanism of anti-invasive activity of RJ on colorectal carcinoma cell line *via* inhibition of Vimentin and Snail protein expression.

Keywords: immunofluorescence; SW-480 cell line; Transwell; apitherapy; transcription factor

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1. Introduction

Colorectal cancer (CRC) is a malignant type of tumor of the gastrointestinal tract with a very high incidence. This disease is ranked as second type of malignant tumors in male and third in females [1].

Important property of cancer, including CRC, is acquisition of invasive potential. Invasive or metastatic phenotype are initiated during epithelial-mesenchymal transition (EMT). This complicated cellular process implies loss of epithelial characteristics, gaining the mesenchymal properties and reorganization of the cytoskeletal architecture which enable motility of cancer cells [2]. Furthermore, these transformed cancer cells secrete proteolytic enzymes which digest the extracellular matrix and lead to destruction of colon wall layers [2,3]. Snail has been recognized as core regulator orchestrating the EMT in CRC, and overexpression of this transcription factor is driving the upregulation of EMT effectors, such as proteolytic enzymes and vimentin. Vimentin is involved in cytoskeletal interaction, cell adhesion, and is prominent EMT marker highly expressed in invasive cancer cells [4].

Therefore, when invasion occurs, metastatic process begins, and when this happens cancer treatment becomes particularly problematic. Hence, science is turning to natural products that can ameliorate standard chemotherapeutical approaches in combating aggressive behavior of CRC [3].

It is known that dietary habits play one of the important roles in elevating risk of CRC initiation and progression [5]. Almost half of colorectal cancer cases can be prevented by altering dietary patterns, therefore it is necessary to pay attention to the food quality and food consumption behaviour [6].

Royal jelly is well known bee product that has been traditionally used for centuries because of its valuable nutritive characteristics and is considered nowdays as effective

dietary supplement. It possesses significant pharmacological properties exerting beneficial effects on human wellbeing [5]. Moreover, it already showed remarkable anti-cancer activity *in vitro* and *in vivo* [7,8], proved to affect invasion of cancer cells and modulating the metastasis of various cancer types [8,9]. Nevertheless, understanding the molecular mechanisms underlying these RJ'a notable actions, especially how this natural product inhibit invasive properties of colorectal cancer cells, is important for the development of optimized therapeutic strategies necessary for combating CRC.

Hence, we aimed to investigate royal jelly's effects on invasiveness of colorectal carcinoma cell line SW-480 and to elucidate the molecular mechanism of this action.

2. Materials and Methods

Royal jelly sample (RJ) was obtained from beekeepers located in Serbia (Central region) produced by *Apis mellifera* L. bee species. RJ was diluted in Phosfate buffer solution to obtain stock solution, and furtherly diluted in complete cell culturing medium (Dulbecco's Modified Eagle Medium supplemented with 10% of Fetal Bovine Serum and penicillin/streptomycin). Two working concentrations 10 and 100 μg/mL were applied and all the following analysis were done 24 h after treatment. Colorectal carcinoma cell line SW-480 was purchased from American Type Culture Collection (ATCC, Manassas, USA) and propagated according to standard culturing procedure (5% CO_2/37°C/humified atmosphere).

Invasive potential of RJ on SW-480 cells was done by applying Transwell assay with collagen layer, simulating extracellular matrix, and protocol for this assay is described in detail previously in Jovanovic et al. (2022). Resulting absorbances were read at 595 nm wavelenght on Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific, USA). Results are presented from three independent experiments performed in triplicates as invasive index (%).

Furthermore, immunofluorescent technique was used to evaluate the protein expression of proinvasive markers Vimentin and Snail, according to the protocol already described in detail (Jovanovic et al., 2022). Micrographs were taken on a inverted fluorescent microscope Eclipse Ti (Nikon Instruments Inc., Tokyo, Japan) at 600× magnification. Obtained micrographs were used for quantification of relative fluorescence intensity following the method described earlier by Schneider et al. (2012), using the ImageJ software package. Results were acquired from three independent experiments performed in triplicates and represented as relative fluorescence per cell (%).

Statistical analysis was done using One-way Anova test within IBM SPSS statistical software package (NY, USA).

3. Results

3.1. RJ suppresses invasiveness

The invasive potential of control and treated SW-480 cells was investigated 24 h after treatment with RJ using Transwell technique. We observed responsiveness of this tested carcinoma cell line to applied treatment. Namely, as it can be observed on Figure 1, both applied RJ concentrations showed inhibitory effect on invasiveness of SW-480 cells. Moreover, this antiinvasive activity of RJ was showed to be dose-dependent, meaning that higher RJ dose (100 μg/mL) showed slightly stronger suppressive effect (Figure 1).

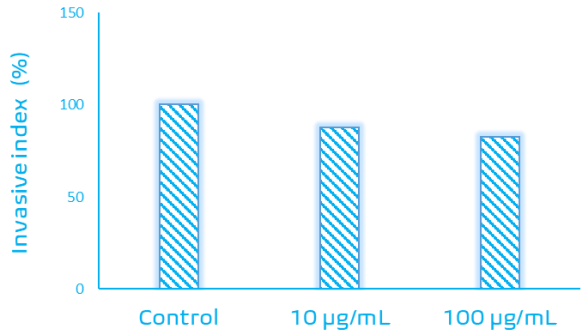
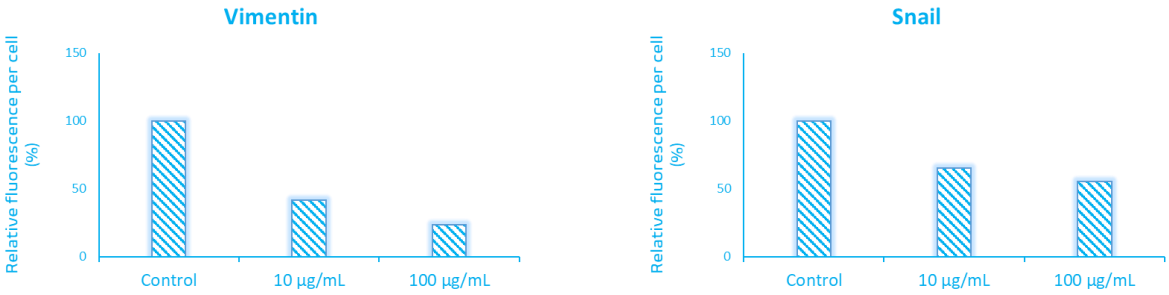


Figure 1. Invasive potential of SW-480 cells 24 h after treatment with two selected RJ concentrations.

3.2. RJ inhibited expression of Vimentin and Snail

The protein expression of cytoskeletal protein Vimentin and transcription factor Snail was investigated by immunofluorescent method. As it can be observed on Figure 1, RJ treatment was able to lower the protein expression of Vimentin and Snail in SW-480 cells after 24 h. The observed decrease was more notable regarding Vimentin expression, than expression of Snail. However, this inhibitory effect was dose-dependent regarding both investigated proteins.

SW-480 cell line showed sensitivity to applied treatment and the higher RJ concentration showed the most prominent suppressive activity regarding both investigated markers. This significant decrease of Vimentin and Snail expression is in correlation with RJ's antiinvasive property, as it was shown previously (Figures 1 and 2).



(**a**) (**b**)

Figure 2. Relative fluorescence intensity of target proteins: Vimentin (**a**) and Snail (**b**) measured in SW-480 cells 24 h after treatment with RJ. Results are presented as changes compared to control values.

Considering these suppressive effects of RJ on protein expression of these two different proteins, we aimed to determine the possible change of Vimentin and Snail location within SW-480 cells.

Obtained micrographs, presented on Figure 3a, show obvious changes in localization of Vimentin in cells treated with RJ, when compared to control cells. Namely, we observed this protein in both cell nuclei and cytoplasm, while in treated cells, its expression was restricted to cell cytoplasm area only (Figure 3a).

When it comes to Snail localization, this transcription factor was present in cell nuclei and cytoplasm in untreated SW-480 cells indicating abundance of this transcription factor. Meanwhile, when cells were treated with RJ, the expression of Snail was gradually inhibited in cytoplasm, and was eventually limited to cell nuclei only in treatment with higher RJ concentration (Figure 3b).

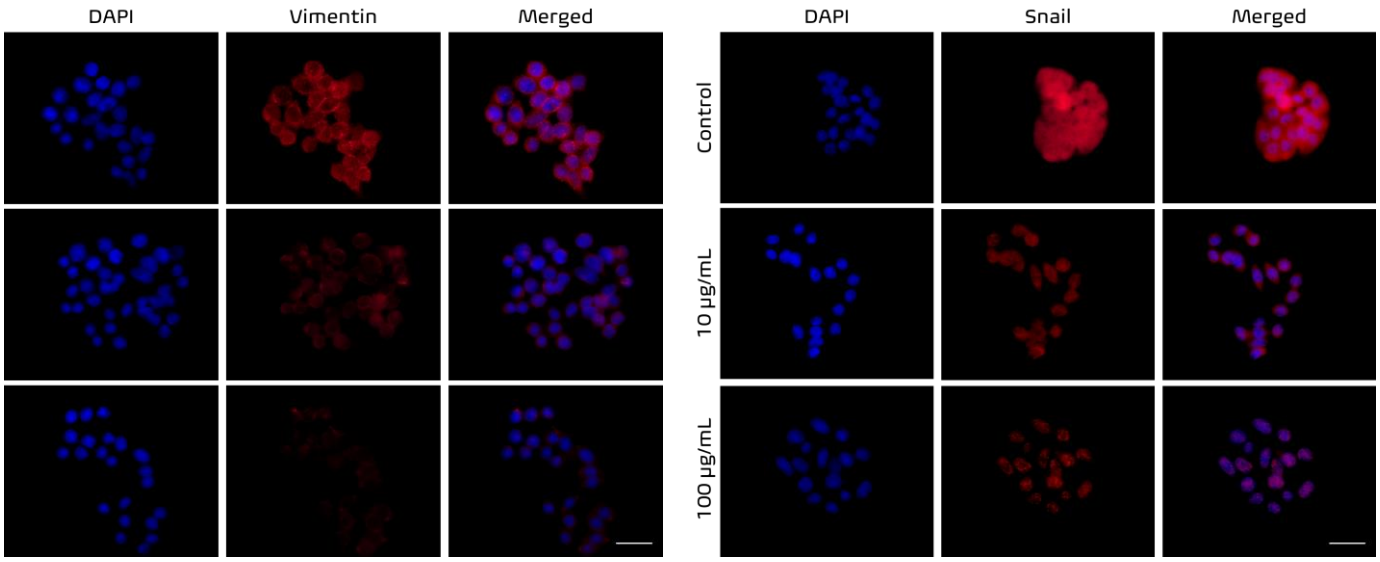


Figure 3. Representative micrographs of Vimentin (a) and Snail (b) protein expression in control and SW-480 cells treated with two selected RJ concentrations. Cell nuclei are stained blue (DAPI dye), while target proteins are stained red (secondary antibody conjugated with Cy3). Scale bar: 50 µm.

4. Discussion

Many studies proved RJ as an effective anti-cancer agent, exerting anti-proliferative, pro-apoptotic, anti-metastatic activity, as well as affecting the expression of cancer-related molecules in patients with cancer [8]. Also, RJ has been used for a long time as a dietary supplement, beacuse of its multiple beneficial effects on human well-being [5]. The analysis of RJ's chemical composition has shown that this natural product consists of many bioactive components, including unsaturated fatty acid 10H2DA, to which the majority of RJ's properties has been attributed to [5,8]. However, only few studies provided scientific explanations for some of these RJ's properties. However, although RJ showed prominent anti-invasive and anti-metastatic activity, the mechanisms underlying these activities still remain unknown. Therefore, present study aimed to provide more detailed experimentally proved data on its anti-invasive regulatory mechanism of action.

According to our presented results, it can be concluded that the exerted effects of RJ on suppression of invasive properties of SW-480 cells were obviously due to reduced expression of Vimentin and Snail.

Since Vimentin, as part of cell cytoskeletal structure, regulate cell movement, it is obvious that insufficient presence of this protein in tested colorectal carcinoma cell line induced by the presence of RJ, caused weaker cell movement through collagen layer. It is known that in cells with induced EMT, the transcription factor Snail is overexpressed and its regulatory role imply concomitant upregulation of Vimentin [10]. Hence, the reduction of Snail caused by applied RJ treatment in SW-480 cells was followed by downregulation of Vimentin, and reduced invasive potential of these cells. We already showed in Jovanovic et al. [5] the inhibitory effect of RJ's unsaturated fatty acid 10H2DA on Snail and Vimentin expression at gene and protein level, which is the explanation for this significant RJ's effect.

However, more detailed investigations are needed in order to consideration of the efficacy and safety, as well as clinical utility of RJ, or various therapies that include RJ, as complementary bioactive substance for application in patients with CRC.

5. Conclusions

Our report indicate the possible molecular mechanism of antiinvasive activity of RJ on colorectal carcinoma cell line. This bee product showed pronounced and promising effects on carcinoma cells *in vitro*, however, our future research will focus on more detailed studies regarding effects of this natural product on invasion of CRC cells.

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