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# The influence of royal jelly and human interferon-alpha (HuIFN-αN3) on proliferation, glutathione level and lipid peroxidation in human colorectal adenocarcinoma cells *in vitro*

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Among royal jelly's (RJ) various biological activities, its possible antitumour activity deserves particular attention. The purpose of this study was to investigate the influence of RJ, its bioactive component 10-hydroxy-2-decenoic acid (10-HDA), and human interferon-alpha (HuIFN-αN3) on the proliferation of human colorectal adenocarcinoma cells (CaCo-2), and ascertain their effect on intracellular glutathione (GSH) level and lipid peroxidation. We studied the antiproliferative (AP) activity of RJ [(0.1 g/10 mL phosphate buffer saline (PBS)], HuIFN-αN3 (1000 I.U. $mL^{-1}$), 10-HDA at 100.0 μmol $L^{-1}$, and their different combinations, in the ratio 1:1, 1:2, and 2:1 on CaCo-2 cells. The GSH level was measured by glutathione assay. The lipid peroxidation was measured by malondialdehyde (MDA) assay. Single RJ had a low AP activity: 2.0 (0.5 mg $mL^{-1}$). HuIFN-αN3 had an AP activity of 2.5 (208.33 I.U. $mL^{-1}$), while 10-HDA had an AP activity of 1.5 (37.5 μmol $mL^{-1}$). The highest AP activity of 3.8 was obtained when RJ and HuIFN-αN3 were applied at the ratio 2:1. In that combination the level of GSH was 24.9±2.4 nmol $g^{-3}$ of proteins (*vs*. 70.2±3.2 nmol $g^{-3}$ in the control) and the level of MDA was 72.3±3.1 nmol $g^{-3}$ (*vs*. 23.6±9.1 nmol $g^{-3}$ in the control). It is generally assumed that 10-HDA, an important constituent of RJ, together with HuIFN-αN3, is responsible for the inhibition of CaCo-2 cells proliferation *in vitro*. In our study, however, RJ and HuIFN-αN3 applied at 2:1 decreased the level of GSH the most and significantly increased lipid peroxidation via MDA in CaCo-2 cells. Future studies should show whether these GSH- and MDA-related activities of RJ, HuIFN-αN3, 10-HDA, and their combinations may decrease the tumorigenicity index and tumorigenic potential of various tumour cells *in vitro*.

KEY WORDS: *antiproliferative activity; antitumour activity; CaCo-2 cells; 10-hydroxy-2-decenoic acid; malondialdehyde*

Royal jelly (RJ) is a milky material secreted by the hypopharyngeal and mandibular glands of young worker bees between the sixth and twelfth day of their life. It is food exclusively for the queen honey bee (*Apis mellifera*) larva, which leads to the development of a sexually mature queen bee (1, 2). Chemically, RJ comprises water (50-60 %), different proteins (18.0 %), carbohydrates (15.0 %), lipids (3-6 %), mineral salts (1.5 %), and vitamins, together with a large number of bioactive substances, such as 10-hydroxy-2-decenoic (10-HDA) acid (3). RJ exhibits various immunomodulatory and antiproliferative properties, as well as possible antitumour activity. It also contains different antibacterial proteins like 350-kDa protein stimulating the proliferation of human monocytes (4-6). In addition, the RJ $Protein_{30}$ fraction exhibits a clear cytotoxic effect on HeLa cells by decreasing the initial cell population by 50 % at the end of treatment (7).

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HuIFN-αN3, a type I interferon, is a multisubtype protein (8, 9) with antiviral, antiproliferative, and antitumour activity (10). It has been used clinically in the treatment of various cancers for over 30 years (11). The molecular mechanism behind its cytoreductive action is still not clear. The antiproliferative effect of HuIFN-αN3 plays a central role in its chemotherapeutic effect. Recent research (12) has also indicated its action in apoptosis pathways as a possible anti-tumour mechanism. It was also shown that HuIFN-αN3 can exert a direct cytotoxic effect on different malignant cells and tumour cell lines *in vitro* (13).

Glutathione (GSH), a low molecular weight thiol, has a central role in the control of the cellular thiol/disulphide redox state, bearing significance for normal redox signalling in cells, among them also in the CaCo-2 cells used in this study (14). Intracellular GSH concentration, cell proliferation, and apoptosis are mutually connected. A high level of GSH enhances cell proliferation, while GSH

depletion results in the inhibition of CaCo-2 cell growth and proliferation, due to the increase of apoptosis.

Lipid peroxidation, or oxidative degradation of lipids, is a process by which free radicals "steal" electrons from the lipids in cellular membranes inducing cell damage. It proceeds by a free radical chain reaction mechanism (15, 16). The end-products of lipid peroxidation are reactive aldehydes with carcinogenic potential. Among them, the most important is malondialdehyde (MDA), a major bioactive marker of lipid peroxidation that exerts numerous biological activities resembling activities of reactive oxygen species.

The aim of this study was to investigate the effect of RJ and 10-HDA on HuIFN-αN3-induced inhibition of CaCo-2 cells proliferation *in vitro* and ascertain their effect on the intracellular level of GSH and lipid peroxidation via MDA activity. We assumed that such an approach might serve to establish possible antiproliferative/antitumor mechanisms in human colorectal adenocarcinoma cells, which could be of value for developing future anticancer treatments based on the use of these bioactive compounds.

## METHODS

### *Material*

The following materials were used: Human Interferon – αN3, (HuIFN-αN3) (Institute of Immunology, Zagreb, Croatia) applied at 1000 I.U. $mL^{-1}$, which was a standard concentration previously used in experiments (17). Royal jelly-fresh (Mižigoj, Ljubljana, Slovenia) (RJ-F(M)) (MEDEX d.o.o., Ljubljana, Slovenia), was applied at 0.1 g/10 mL (18). 10-hydroxy-2-decenoic acid (10-HDA) (Sigma-Aldrich, Missouri, USA) was applied at 100 μmol $L^{-1}$ (19). All of the reagents were dissolved in the phosphate buffer saline (PBS), pH=7.2 and then filtered through a 0.2 μm syringe filter (Millipore, USA).

### *Cell culture*

CaCo-2 cells (Institute for Microbiology and Immunology, Ljubljana, Slovenia) were cultivated in Eagle's medium with L-Glutamine (2.0 mmol $L^{-1}$) (Sigma-Aldrich, Missouri, USA) and antibiotics: Penicillin (100 units $mL^{-1}$), Streptomycin (100 μg $mL^{-1}$), and Gentamycin (50 μg $mL^{-1}$) (Sigma-Aldrich, Missouri, USA) and supplemented with 10 % of foetal calf serum (FCS) (Sigma-Aldrich, Missouri, USA). Before the experiment, the cells were multiplied and their viability and capability for proliferation was assayed by the MTT Cell Proliferation Assay Kit (K299-100) (BIOVISION, Milpitas, California, USA). The cell cultivation was performed in 96-well flat microtiter plates in 5 % $CO_2$ at 37 °C or in 25 $cm^2$ flasks (Sterilin, Sigma-Aldrich, Missouri, USA) in 5 % $CO_2$ at 37 °C.

### *Design of the study*

The experiments were designed as follows: single substances: RJ-F(M), HuIFN-αN3 and 10-HDA in the previously stated concentrations, were added alone in a volume of 100 μL/well. When different combinations between them were used, for the ratio 1:1 100+100 μL/well was added. For the ratio 1:2, 66.8+133.2 μL/well and for the ratio 2:1, 133.2+66.8 μL/well was added.

### *Antiproliferative (AP) activity*

Single substances or their combinations, as described in the design of the study, were added in a volume of 200 μL per well in the first well in row of 96-well flat microtiter plates, and the samples were serially transferred per 100 μL from 1:2 to 1:4096 in Eagle's medium with L-Glutamine and antibiotics. After the substances, the cells (CaCo-2) were added ($10^4$ cells/well/100 μL) in Eagle's medium with L-Glutamine and antibiotics and 10 % FCS. The cells without substances were added separately (the negative control). As positive controls, single tested substances (RJ, HuIFN-αN3, 10-HAD) were used. The microtiter plates were incubated for 72 hours at 37 °C in a 5 % $CO_2$ atmosphere. Afterward, the supernatants were discharged and the cells were fixed with the addition of 100 μL/well of 10 % formalin (Sigma-Aldrich, Missouri, USA) in PBS. After two hours, the fixative was removed and the cells were washed twice with the PBS. After that, 2 % Rhodamine B (Sigma-Aldrich, Missouri, USA) (100 μL/well) was added for 15 minutes. This was then removed, and the cells were washed twice with PBS and air-dried. On the dried plates, the optical density (OD) at 550 nm was measured (Synergy HTX Multi Mode Reader with Gen 5 software, Biotek, Winooski, USA). The AP activity was determined with the well in rows where 50 % cell growth inhibition was found. The $AP_{50}$ was calculated for each separate substance (RJ, HuIFNαN3, and 10-HDA) (20).

### *Glutathione determination*

CaCo-2 cells were cultivated in 25 $cm^2$ flasks (Sterilin, Sigma-Aldrich, Missouri, USA) in Eagles' medium with L-Glutamine and antibiotics and 10 % FCS. When monolayers were developed, the cells in flasks were treated with substances alone or their combinations in a volume of 1.0 mL when single substances were added, and a total of 2.0 mL when the combinations were added. The cells were treated for 24 hours at 37 °C and 5% $CO_2$. The medium was removed, and the cells were detached with trypsin (Sigma-Aldrich, Missouri, USA) and treated with 1 mL of 10 mmol $mL^{-1}$ Tris-HCl solution (pH=6.0) containing 0.5 mol $mL^{-1}$ diethylene triamine pentacetic acid (DTPA) (Sigma-Aldrich, Missouri, USA), and syringed several times with an insulin syringe for their lysis. The cell protein amount was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA) and Bovine serum albumin (BSA) (Sigma-Aldrich, Missouri, USA) as a

standard. For total GSH determination, 100 µL of DL-Dithiothreitol (DTT), 25 µmol $L^{-1}$, and 150 µL of 0.1 mol $mL^{-1}$ Tris-HCl (pH 8.5) were added to 50 µL of the cell lysate. After 30 minutes on ice, the proteins were precipitated by adding 750 µL of 2.5 % (wt./vol) 5-sulfosalicilic acid and centrifuged at 13000 *g* (Centric, Tehtnica d.o.o., Železniki, Slovenia) for 4 minutes at 4 °C. The cellular supernatants were used in Glutathione Assay Kit (Sigma-Aldrich, Missouri, USA) to measure the GSH level at OD at 412 nm (Synergy HTX Multi Mode Reader with Gen 5 software, Biotek, Winooski, USA) and expressed as nmol of GSH $g^{-3}$ of proteins.

*Measurements of lipid peroxidation*

CaCo-2 cells were cultivated in 25 $cm^2$ flasks (Sterilin, Sigma-Aldrich, Missouri, USA) in Eagles' medium with L-Glutamine and antibiotics and 10 % FCS. When the monolayer was formed, the cells were treated with substances alone or their combinations as described in the design of the study, in a quantity of 1.0 mL when single substances were added, and a total of 2.0 mL when the combinations were added. The treated/non-treated cells were incubated for 24 hours at 37 °C and 5 % $CO_2$. The medium was removed and cells detached with trypsin washed and resuspended in 5 mL of PBS. Cell protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA) and BSA as a standard. A measure of 1 mL of thiobarbituric acid (TBA) reagent (0.38 % 2-TBA, 15.0 % TBA, 0.3 mol $mL^{-1}$ HCl) was added to the cell suspension. The samples were heated at 95 °C for 20 minutes, chilled to room temperature and centrifuged at 1500 *g* for 10 minutes. The TBA reactive substances (RS) developed by lipid peroxidation were measured in the supernatant at OD at 535 nm (Synergy HTX Multi Mode Reader with Gen 5 software, Biotek, Winooski, USA), according to the TBA method (21, 22). The results were expressed as MDA nmol $g^{-3}$ of protein.

*Data analysis*

T-test was used for significance determination (*$p$<0.1, **$p$<0.05); data are shown as mean value±standard deviation. Each of the tests was performed in triplicate and each experiment was repeated three to four times.

## RESULTS AND DISCUSSION

*Antiproliferative activity*

The following results of AP activity and concentrations at $AP_{50}$ for single substances were obtained: RJ: 2.0 (0.5 mg $mL^{-1}$), HuIFN-αN3: 2.5 (208.33 I.U. $mL^{-1}$) and 10-HDA: 1.5 (37.5 µmol $L^{-1}$). The observed AP activity was relatively low. The AP activity of their combinations (1:1, 1:2, 2:1) is shown on Figure 1.

The highest AP activity was obtained when the combination of RJ-F (M) and HuIFN-αN3 was used in a 2:1 ratio. In this case the AP activity was 3.8. When the combination of the RJ-F (M) component 10-HDA and HuIFN-αN3 2:1 was tested, the AP activity was 2.4-2.6, which was much lower than with RJ-F (M) and HuIFN-αN3. Such results suggest that RJ-F (M) could contain some

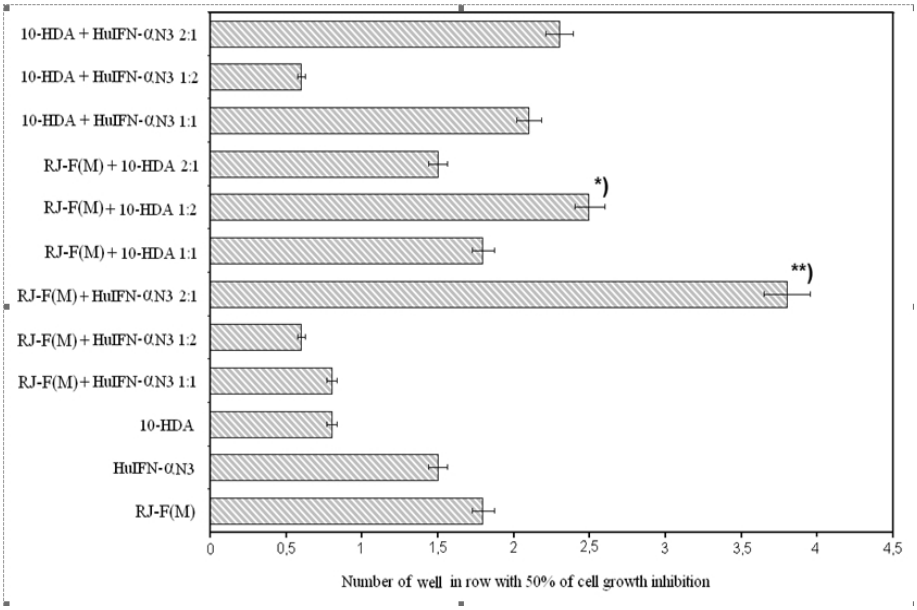


**Figure 1** *The AP effect of RJ-F (M), HuIFN-αN3, 10-HDA and their combinations (1:1, 1:2 and 2:1) on the CaCo-2 cell line. Inhibition of proliferation of CaCo-2 cell line treated with RJ-F (M) (0.1 g /10 mL PBS), HuIFN-αN3 (1000 I.U. $mL^{-1}$), 10-HDA (100 µmol $mL^{-1}$) and different combinations between them (1:1, 1:2 and 2:1) was analysed. The AP activity was determined by Rhodamine B staining method after 72 h of incubation at 37 °C and 5 % $CO_2$. The t-test was used for significance determination (*p<0.1, **p<0.05); data are shown as mean value ± standard deviation*

other components responsible for the relatively strong AP activity of the combination RJ-F (M) and HuIFN-αN3 in the ratio 2:1. In this respect, the possible role of the RJ Protein$_{30}$ water soluble fraction that previously exhibited the clear cytotoxic effect on HeLa cells by decreasing the initial cell population by 50 % at the end of treatment (7) should be also anticipated.

It is known that the AP activity of the RJ-F (M), HuIFN-αN3, 10-HDA on the CaCo-2 cells is connected with the induction of apoptosis and cytotoxicity (14). Also their influence on the glutathione level and lipid peroxidation was found (23).

*GSH determination and measurement of lipid peroxidation*

The results obtained in our study show that RJ-F (M), HuIFN-αN3, 10-HDA and their combinations decreased the level of glutathione and increased the lipid peroxidation via the MDA. Detailed data are shown in Table 1.

Glutathione (GSH) plays an important role in many cellular processes, like cell differentiation, proliferation, and apoptosis and cancer. While GSH deficiency, or a decrease in the GSH/glutathione disulphide (GSSG) ratio, leads to an increased susceptibility to oxidative stress in the progression of cancer, elevated GSH levels increase the antioxidant capacity and resistance to oxidative stress found in cancer cells. It is important to stress that RJ-F (M), HuIFN-αN3, 10-HDA, and their combinations decreased the level of glutathione and significantly increased the lipid peroxidation via the MDA.

Some of the mechanisms of the possible antitumour mechanisms of royal jelly are connected with the modulation of the oxidative stress and induction of apoptosis. (24). Practically the same effects were found after the treatment of the Pancreatic Cancer Cell tumour cells PaCa-44 with 10-HDA, where the induction of the apoptosis was found (25). It is interesting to note that the antitumour activity of HuIFN-αN3 was also connected with the induction of apoptosis and the modulation of the oxidative stress in rats with breast cancer (26).

The most important finding of our study is that royal jelly, especially its constituent 10-HDA and HuIFN-αN3 had similar active points in the antitumor activity, which in proper ratio can be enhanced. It can be concluded, that the most active was combination of RJ-F(M) and HuIFN-αN3 2:1, where the level of the GSH was 24.9±2.4 nmol g$^{-3}$ of proteins (*vs*. 70.2±3.2 nmol g$^{-3}$ in the control) and the level of MDA 72.3±3.1 nmol g$^{-3}$ (*vs*. 23.6±9.1 nmol g$^{-3}$ in the control).

Future experiments will show whether these GSH- and MDA- related activities of RJ-F (M), HuIFN-αN3, 10-HDA and their combinations may cause the decrease of the tumorigenicity index of different tumour cells *in vitro*, as previously reported in the literature (27, 28), also through their tumorigenic potential. This is important for the practical use of royal jelly (10-HDA) and HuIFN-αN3 in a combination that could be of value for future development of tumour therapy based on the use of bioactive compounds.

*Conflict of Interests*

All the authors declare no conflict of interest.

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**Table 1** *Glutathione (GSH) determination and measurement of lipid peroxidation (MDA) after the CaCo-2 cells treatment with RJ, HuIFN-αN3, 10-HDA and their combinations: 1:1, 1:2 and 2:1*[a]

| Sample | Glutathion (GSH) [b] | Malondialdehyde (MDA)[c] |
|---|---|---|
| | (Mean±SE) | (Mean±SE) |
| Cell control | 70.2±3.2 | 23.6±9.1 |
| RJ-F(M)[d] | 43.8±2.8 | 30.2±4.3 |
| HuIFN-αN3 | 28.7±6.4 | 38.6±4.2 |
| 10-HDA[e] | 33.6±5.8 | 50.7±4.6 |
| RJ-F(M)+HuIFN-αN3 1:1 | 45.2±4.7 | 43.6±4.1 |
| RJ-F(M)+HuIFN-αN3 1:2 | 40.8±3.1 | 58.3±5.2 |
| RJ-F(M)+HuIFN-αN3 2:1 | 24.9±2.4** | 72.3±3.1** |
| RJ-F(M)+10-HDA 1:1 | 40.6±4.5 | 43.1±2.6 |
| RJ-F(M)+10-HDA 1:2 | 37.2±2.1 | 50.6±4.5 |
| RJ-F(M)+10-HDA 2:1 | 30.3±3.7* | 61.6±5.2* |
| 10-HDA+HuIFN-αN3 1:1 | 29.5±1.7 | 49.6±4.2 |
| 10-HDA+HuIFN-αN3 1:2 | 42.6±5.3 | 57.2±2.6 |
| 10-HDA+HuIFN-αN3 2:1 | 25.6±3.1 | 55.6±6.2 |

[a]*t-test was used for significance determination;* * p<*0.1,*** p<*0.05*
[b]*Measured as nmol g$^{-3}$ of proteins;* [c]*Measured as nmol g$^{-3}$ of proteins;* [d]*RJ-F(M)=royal jelly – Fresh(Mižigoj);* [e]*10-HDA=10-hydroxy-2-decenoic acid*

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**Vpliv matičnega mlečka in humanega interferona-alfa (HuIFN-αN3) na proliferacijo, nivo glutationa in na preoksidacijo lipidov v humanih kolorektalnih adenokarcinomskih celicah *in vitro***

Kot del biološke aktivnosti MM (Matičnega mlečka) so avtorji preučevali njegovo protitumorsko delovanje kot tudi možno interakcijo s humanim interferonom alfa (HuIFN-αN3). Cilj opravljenih poskusov je bil preučiti vpliv kombinacije med MM in HuIFN-αN3 na proliferacijo celic Humanega kolorektalnega adenokarcinoma (CaCo-2) in njun vpliv na znotrajcelični nivo glutationa (GSH) in peroksidacijo lipidov. Avtorji so preučevali AP (Antiproliferativno) delovanje MM (0.1 g/10 mL fosfatnega pufra) (PBS), HuIFN-αN3, (1000 I.U. $mL^{-1}$), 10-hidroxy-2-decenoične kisline (10-HDA) (100.0 μmol $L^{-1}$) in različne kombinacije med njimi (1:1, 1:2 in 2:1) na celice CaCo-2 *in vitro*. Njihov vpliv na znotrajcelični nivo GSH so merili s pomočjo komercialnega kita. Peroksidacijo lipidov so merili s pomočjo meritve vrednosti malondialdehida (MDA). MM sam kaže AP aktivnost 2.0 (0.5 mg $mL^{-1}$ ). HuIFN-αN3 ima AP aktivnost 2.5 (208.33I.U.$mL^{-1}$) medtem ko ima 10-HDA AP aktivnost 1.5 (37.5 μmol $mL^{-1}$). AP aktivnost kombinacije MM:HuIFN-αN3 (2:1) je bila 3.8. Pri tej kombinaciji je bil viden vpliv na nivo GSH: 24.9±2.4 nmol $g^{-3}$ proteinov (70.2±3.2 nmol $g^{-3}$ pri kontroli). Nivo MDA je bil 72.3±3.1 nmol $g^{-3}$ pri kontroli). 10-HDA je glavna sestavina MM, ki v kombinaciji s HuIFN-αN3 deluje antiproliferativno na CaCo-2 celice. MM in HuIFN-αN3 v kombinaciji 2:1 pospešujeta peroksidacijo lipidov (MDA) in zmanjšujeta nivo glutationa (GSH). Nadaljni poskusi bodo pokazali ali z GSH- in MDA- povezane aktivnosti MM, HuIFN-αN3, 10-HDA in kombinacij med njimi, zmanjšujejo indeks tumorigenosti in s tem tumorigeni potencijal različnih tumorskih celic *in vitro*.

KLJUČNE BESEDE: *antiproliferativno delovanje; CaCo-2 celice; protitumorsko delovanje; 10-hidroksi-2-decenoična kislina; malondialdehidi*