

EFFECT OF ROYAL JELLY(RJ) ON HUMAN INTERFERON-Alpha (HuIFN- α) INHIBITION OF HUMAN COLON CANCER CELLS (CaCo-2) PROLIFERATION *IN VITRO*

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ABSTRACT

Background. Royal jelly is a milky material secreted by the pharyngeal glands of young worker bees, which when fed as a sole nutrient to larvae causes the development of sexual mature queen bees. The unusual nature of this material has prompted different investigations into its biological/pharmacological/microbiological properties. As a part of the biological activity of royal jelly, an investigation of its possible antitumor activity was performed. Interferon-Alpha (HuIFN- α) was used clinically in the treatment of different cancers but the molecular mechanism behind its cytoreductive action is still unknown. The anti-proliferative effect of HuIFN- α was suggested to be the main factor in its antitumor activity. The experiments presented were aimed to investigate the effect of royal jelly on HuIFN- α induced inhibition of human colon cancer cells proliferation *in vitro*, and the effect on intracellular Glutathione (GSH) and Lipid peroxidation (MDA – assay)

Material and methods. Fresh Royal jelly (Medex d.d.), (0.1g/10 ml PBS(Phosphate buffer saline)) was used; Human Interferon-Alpha (HuIFN- α) (1000 I.U./ml) was from Sigma-Aldrich, 10-HDA (100 μ M /ml) (10-Hidroxy-2-decenoic acid) was from Sigma-Aldrich. Experiments were performed on CaCo-2 cells cultivated in Eagle's medium+10% of FCS and antibiotics (Penicilline, Streptomycine, Gentamycine). The anti proliferative (AP) activity was measured with number of the well with cca. 50% cell growth inhibition. The concentrations at AP₅₀ were determined. Cells were treated as follows: (a) Royal jelly, (b) HuIFN- α , (c) 10-HDA, (č) Royal jelly + IFN α 1:1, 1:2, 2:1, (d) Royal jelly + 10-HDA 1:1, 1:2, 2:1. The effect on the Glutathion level was measured by Sigma-Aldrich glutathione assay kit. The lipid peroxidation was measured by MDA (Malondialdehyde) assay.

Results. The following results of AP activity and concentrations at AP₅₀ were obtained: (1) Royal jelly: 2,0(0,005)g/ml), (2)HuIFN- α : 2,5 (208,33 I.U./ml)(3) 10-HDA: 1,5(37,5 μ M/ml) ; (4) Royal jelly+HuIFN- α 1:1 0,9, 1:2 0,9, 2:1 3,9; (5) Royal jelly + 10-HDA: 1:1 1,8,1:2 1,5, 2:1 3,2; (6)HuIFN α + 10-HDA: 1:1 1,8, 1:2 1,5, 2:1 3,2. The RJ-F(M), HuIFN- α and 10-HDA and their combinations decrease the level of Glutathione and increase the lipid peroxidation via the MDA

Conclusions. From the presented experiments it can be concluded: (1) Royal jelly alone, has low AP activity: 1.0 (0,01g/ml) ; (2) HuIFN- α alone, has AP activity of 2.5 (208,33 I.U./ml) (3) The optimal combination between Royal jelly and HuIFN- α was 2:1 where the AP activity was 3.5. (4) It seems that the 10-Hidroxy-2-decenoic acid, as the main component of the Royal jelly is responsible for the influence of royal jelly on Interferon's Alpha (HuIFN- α) inhibition of Human colon cancer cells (CaCo-2) proliferation *in vitro*. The most active was the combination of RJ-F(M) and HuIFN- α 2:1, where the level of GSH was 24,9 \pm 2,4

nmol/mg of proteins ($70,2 \pm 3,2$ nmol/mg in Control) and the level of MDA was $72,3 \pm 3,1$ nmol/mg ($23,6 \pm 9,1$ nmol/mg in Control)

Key words: Royal Jelly, Human Interferon – Alpha (HuIFN- α), 10-HDA, CaCo-2 cells, Antiproliferative activity (AP), Glutathione (GSH), Lipid peroxidation (MDA)

INTRODUCTION

Royal jelly (RJ) is a milky material secreted by the hypopharyngeal and mandibular glands of young worker bees between the sixth and twelfth days of their life. It is the exclusive food for the queen honey bee (*Apis mellifera*) larva. Fed as the sole nutrient to larvae, causes the development of sexual mature queen bees. Chemically RJ comprises water (50-60%), different proteins (18%), carbohydrates (15%), lipids (3-6%), mineral salts (1,5%) and vitamins (Nagai, T., Inoue, R. 2004; Ramadan, F.H., Al-Ghamdi, A., 2012) together with a large number of bioactive substances such as: 10-hydroxy-2-decenoic acid with immunomodulating properties, antiproliferative properties and possible antitumor activity, (Townsend, F.G., Morgan, J.F., Tolnai, S. et al. 1960) fatty acids, peptides and different antibacterial proteins like 350-kDa protein which stimulates the proliferation of human monocytes. (Watanabe, K., Shinimoto, H., Kobori, M. et al. 2001). In addition, the RJ Protein₃₀ – fraction showing the clear cytotoxic effect on HeLa cells by decreasing the initial cell population by 50% at the end of treatment. (Salazar-Olivio, L.A., Paz-Gonzalez, V. 2005)

Human Interferon-Alpha (HuIFN- α) is a multi-subtype protein (Bekisz, J., Schmeisser, H., Hernandez, J. et al. 2004; Kalliolias, D.G., Ivashkiv, B.L. 2010) showing antiviral (Garcia-Sastre, A., Biron, A.C. 2006; Sadler, A.J., Williams, R.G.B. 2008), antiproliferative, antitumor (Liu, X., Lu, J., He, M.L. et al. 2013), radioprotective and antitoxic activity. (Thyrell, L., Erickson, S., Zhivotovsky, B. et al. 2002) It has been used clinically in the treatment of a variety of cancers for over 30 years (Jonasch, E., Haluska, G.F. 2001) even the molecular mechanism behind its cyto-reductive action is still not clear. The antiproliferative effect of HuIFN- α plays a central role in its chemotherapeutic effect. Recent research has also indicated its action in the apoptosis pathways as a possible anti-tumor mechanism (Jedema, I., Barge, R.M.Y., Willemze, R. et al. 2003). Different studies have shown that HuIFN- α can exert the direct cytotoxic effect on different malignant cells and tumor cell lines *in vitro*. (Grander, D., Xu, B., Einhorn, S. et al. 1993; Sangfelt, Q., Erickson, S., Castro, J., et al. 1997)

The presented experiments were aimed to elucidate the effect of Royal Jelly (RJ) on Interferon-Alpha (HuIFN- α) induced inhibition of Human Colon Cancer cells (CaCo-2) proliferation *in vitro* and effect on the intracellular level of Glutathione (GSH) and on the Lipid peroxidation via MDA activity.

MATERIAL AND METHODS

1. Material:

During the experiments the following materials were used: (1) Human leukocyte Interferon (HuIFN- α) (Sigma-Aldrich) 1000 I.U/ml, (2) Royal Jelly-Fresh (Mižigoj) (RJ-F(M)) (MEDEX d.d.), 0,1g/10 ml, (3) 10-Hidroxy-2-decenoic acid (10-HDA) (Sigma-Aldrich) (100 μ M/ml). All reagents were dissolved in the sterile PBS (Phosphate Buffer Saline) pH = 7,2 and then filtered through the 0,2 μ m syringe filter (Millipore, USA), (4) CaCo-2 (Colon Cancer Cells) cells were cultivated in Eagle's medium with L-Glutamine and antibiotics (Penicillin, Streptomycin and Gentamycin) and with added 10% of Fetal Calf

Serum (FCS) (Sigma-Aldrich). The cell cultivation was performed in 96well trays or 25cm² flasks (Sterilin, Germany).

2. Methods

2.1. Antiproliferative (AP) activity

In the 96well flat microtiter plates the samples were added (200µl) and they were serially diluted from 1:2 till 1:4096 in the Eagle's medium with L-Glutamine and antibiotics (Penicilline, Streptomycine and Gentamycine). The following substances or their's combinations were added: (a)RJ-F(M), (b) HuIFN- α , (c) 10-HDA, (č) RJ-F(M) + HuIFN- α : 1:1, 1:2, 2:1; (d) RJ-F(M) + 10-HDA: 1:1, 1:2, 2:1; (e) HuIFN- α + 10-HDA: 1:1, 1:2, 2:1 After the substances, the cells (CaCo-2) were added (10⁴ cells/well/100µl) in the Eagle's medium with L-Glutamine and antibiotics (Penicilline, Streptomycine and Gentamycine) and 10% FCS (Fetal calf serum)(Sigma-Aldrich). Separately, the cells without substances before, were added (Cell control). The microtiter plates were incubated for 72^h at 37° C in 5% CO₂ atmosphere. After three days, the supernatants were discharged, and cells were fixed with the addition of 100µl/well of 10% formaline in PBS (Phosphate buffer saline). After two hours the fixative was removed, and the cells were washed twice with the PBS. Afterthat, the 2% Rhodamine B (100 µl/well) was added for 15 minutes. The Rhodamine B was than removed, and the cells were washed twice with PBS and air-dried. On the dried plates the OD at 550 nm were measured. The AP activity was determined with the well in row, where 50% cell growth inhibition was found. (Borden, E.C., Hogan, T.F., Voelkel, G.J. 1982)

2.2. Glutathione determination

CaCo-2 cells were cultivated in 25cm² flasks in Eagles's medium with L-Glutamine and antibiotics (Penicilline, Streptomycine and Gentamycine) and 10% FCS (Fetal calf serum)(Sigma-Aldrich). When the monolayer was developed, the cells (flasks) were treated with: (a)RJ-F(M), (b) HuIFN- α , (c) 10-HDA, (č) RJ-F(M) + HuIFN- α : 1:1, 1:2, 2:1; (d) RJ-F(M) + 10-HDA: 1:1, 1:2, 2:1; (e) HuIFN- α + 10-HDA: 1:1, 1:2, 2:1 for 24 hours at 37 ° C. The medium was removed, and cells were detached with trypsin and treated with 1 ml of 10mM Tris-HCl solution (pH=6,0) containing 0,5 M diethylenetraminopentacetic acid, and syringed several times with insuline syringe for their lysis. The cell protein concenration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, USA) and Bovine serum albumin (BSA) (Sigma-Aldrich) as a standard. For total Glutathion determination, 100 µl of DL-Dithiothreitol (DTT), 25µM and 150 µl of 0,1 M Tris-HCl (pH 8,5) were added to 50µl of the cell lysate. After 30 minutes on ice, the proteins were precipitated by the addition of 750 µl of 2,5% (wt/vol) 5-sulfosalicilic acid and centrifuged at 13.000 g for 4 min at 4° C The celar supernatant was used in the Gluthatione Assay Kit (Sigma-Aldrich) to measure the Gluthatione at 412 nm and express it as nmoles of Gluthation/mg of proteins of sample.

2.3. Measurement of lipid peroxidation

CaCo-2 cells were cultivated in 25cm² flasks in Eagles's medium with L-Glutamine and antibiotics (Penicilline, Streptomycine and Gentamycine) and 10% FCS (Fetal calf serum)(Sigma-Aldrich). When the monolayer occured, the cells (flasks) were treated with: (a)RJ-F(M), (b) HuIFN- α , (c) 10-HDA, (č) RJ-F(M) + HuIFN- α : 1:1, 1:2, 2:1; (d) RJ-F(M) + 10-HDA: 1:1, 1:2, 2:1; (e) HuIFN- α + 10-HDA: 1:1, 1:2, 2:1 for 24 hours at 37 ° C. The medium was removed, and cells were detached with trypsin, washed and resuspended in 5 ml of PBS (Phosphate buffer saline). Cell protein concenration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, USA) and Bovine serum albumin (BSA) (Sigma-Aldrich) as a standard. A measurment of 1 ml of thiobarbituric acid (TBA) reagent (0,375 % 2-TBA, 15% TBA, 0,25 N HCl) was added to the cell suspension. Samples were heated at 95

°C for 20 minutes and then chilled to room temperature and centrifuged at 1500 g for 10 minutes. TBA reactive substances (RS) produced by lipid peroxidation was measured in the supernatant at 535 nm according to the TBA method (Buege, J.A., Aust,S.D., 1978, Devasagayam, T.P.A., Bolor, K.K., Ramasarma, T., 2003). The results were expressed as malondialdehyde (MDA) nmol/mg of protein.

RESULTS AND DISCUSSION

During the experiments the following results were obtained:

1. Antiproliferative activity

The following results of AP activity and concentrations at AP₅₀ were obtained: (a) R J-F(M): 2,0 (0,005g/ml), (b) HuIFN- α : 2,5 (208,33 I.U./ml), (c) 10-HDA: 1,5 (37,5 μ M/ml), (č) RJ-F(M) + HuIFN- α : 1:1= 0,8, 1:2=0,5, 2:1=3,8; (d) RJ-F(M)+10-HDA= 1:1=1,8; 1:2=1,5; 2:1=1,5; (e) HuIFN- α + 10-HDA: 1:1=2,1; 1:2= 0,5; 2:1=2,3. **(FIGURE 1)**

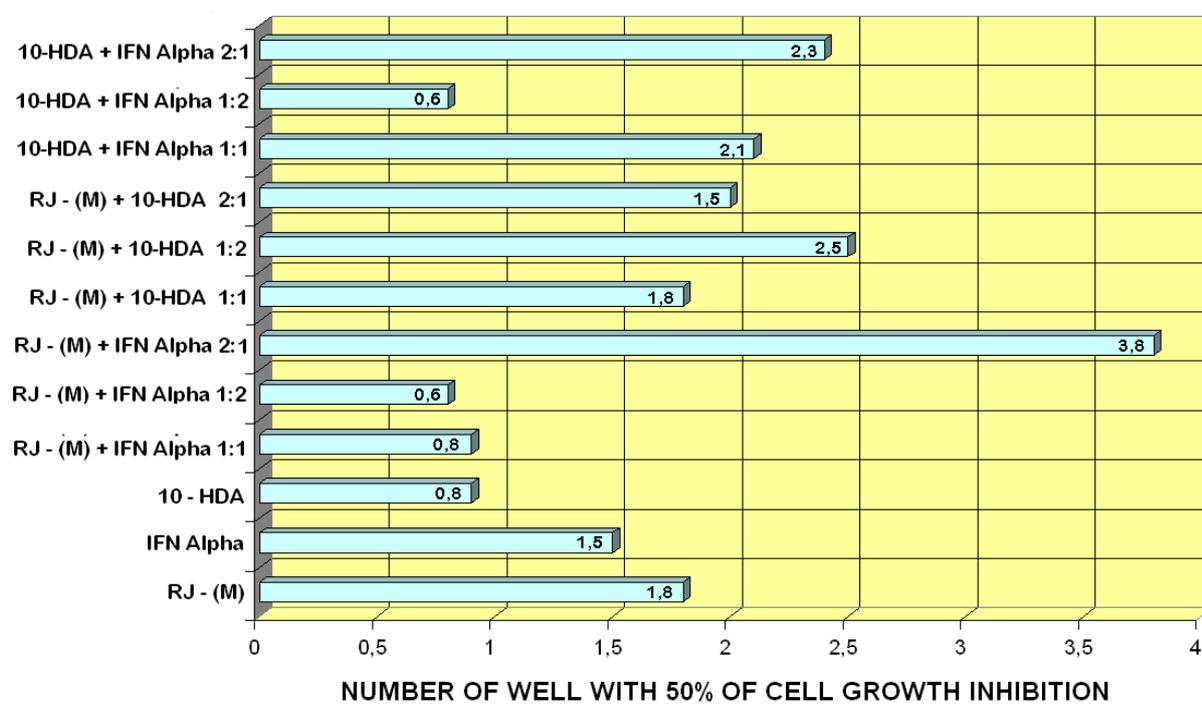


FIGURE 1: Effect of RJ-F(M), HuIFN-Alpha,10-HDA and their's combinations on the proliferation of the CaCo-2 cells.

2. Glutathion (GSH) determination and measurement of lipid peroxidation (MDA)

The following results were obtained during the experiments: (**TABLE 1**)

TABLE 1: Glutathion (GSH) determination and measurement of lipid peroxidation (MDA) after the CaCo-2 cell treatment with RJ-F(M), HuIFN- α , 10-HDA and their's combinations.

SAMPLE:	GLUTATHION (GSH) ¹⁾	MALONDIALDEHYDE (MDA) ²⁾
CELL CONTROL	70,2 ±3,2	23,6±9,1
RJ-F(M)	43,8 ±2,8	30,2±4,3
HuIFN- α	28,7 ±6,4	38,6±4,2
10-HDA	33,6± 5,8	50,8±3,1
RJ-F(M)+HuIFN- α 1:1	45,2± 4,1	43,6±4,1
RJ-F(M)+HuIFN- α 1:2	40,8± 3,1	58,3±5,2
RJ-F(M)+HuIFN- α 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±4,3
RJ-F(M)+10-HDA 2:1	30,3±3,7	61,6±3,2
10-HDA+HuIFN- α 1:1	29,5±1,7	49,6±3,2
10-HDA+HuIFN- α 1:2	42,6±3,2	57,2±2,6
10-HDA+HuIFN- α 2:1	22,6±2,4	55,6±3,2

1) Measured as nmol/mg of proteins; 2) Measured as nmol/mg of proteins;

Inhibition of CaCo-2 cell growth, the AP activity of RJ-F(M), HuIFN- α , 10-HDA and their's combinations shows, that RJ-(M) alone has low AP activity (1,8), with ID50=0,005g/ml; HuIFN- α alone has AP activity 1,5, with ID50= 208,33 I.U./ml. The 10-HDA alone has the AP activity of 0,8 with ID50=37,5 μ M/ml. The best combination between them was the RJ-F(M)+HuIFN-Alpha 2:1, here the AP activity was 3,8. In this respect it seems that the main active component in the RJ-F(M) is the 10-HDA, even the possible role of the RJ Protein₃₀ – fraction that exhibit the clear cytotoxic effect on HeLa cells by decreasing the initial cell population by 50% at the end of treatment.(Salazar-Olivio, L.A., Paz-Gonzalez, V. 2005) shouldn't be neglected. It is known, that the AP activity of the RJ-F(M), HuIFN- α , 10-HDA on the CaCo-2 cells is connected with the induction of apoptosis, possible cytotoxicity and influence on the Glutathion and lipid peroxidation (Traverso, N., Ricciarelli,R.,Nitti,M. et al. 2013). The most important fact is, that RJ-F(M), HuIFN- α , 10-HDA and their's combinations decrease the level of Glutathion and explosively increase the lipid peroxidation via MDA level. The most active was the combination of RJ-F(M)+HuIFN- α 2:1, where the level of the GSH was 24,9±2,4 nmol/mg of proteins (70,2 ±3,2 nmol/mg in Control) and level of MDA was 72,3±3,1 nmol/mg (23,6±9,1 nmol/mg in Control). The further experiments will show if these AP related activities are connected with the cytotoxicity or apoptosis only. (Merendino,N; Loppi,B; D'Aquino,M. Et al. 2005), despite

some data show (Popadić, S., Ramić, Z., Medenica, L. et al. 2009) that LDH analysis of the influence of 10-HDA on adult keratinocytes, excluded the necrosis.

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