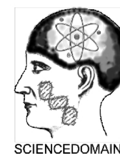
European Journal of Medicinal Plants
3(1): 88-98, 2013

SCIENCEDOMAIN *international*
www.sciencedomain.org



Cytoprotective Potential of Royal Jelly on Human Umbilical Vein Endothelial Cells against Nicotine Toxicity via Catalase

Roongtawan Supabphol[1*] and Athikom Supabphol[2]

[1]Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand.
[2]Department of Surgery, Faculty of Medicine, HRH Princess Maha Chakri Sirindhorn Medical Center (MSMC), Srinakharinwirot University, Ongkharak, Nakorn Nayok, Thailand.

Authors' contributions

This work was carried out in collaboration between two authors. Author RS did the experimental design, performed the cytotoxic, cytoprotective, and catalase activity assays, statistical analysis, and literature searches. Author AS managed for the cell culture, RT-PCR, literature searches, and wrote first draft of the manuscript. Both authors read and approved the final manuscript.

Research Article

Received 28th March 2012
Accepted 25th June 2012
Published 9th December 2012

ABSTRACT

Aims: To examine the cytoprotective effects and mechanisms of a royal jelly extract in protecting the human umbilical vein endothelial cells (HUVECs) from nicotine toxicity.
Study Design: Laboratory experimental tests.
Place and Duration of Study: Department of Physiology and Department of Surgery, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand, between June 2011 and February 2012.
Methodology: Cytotoxic assay of royal jelly to HUVECs was performed by using the 3-(4,5-dimethylthiazol,2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. The cytoprotective effect was then investigated by examining the presence of vacuole-like structures in HUVECs exposed to nicotine 5 or 7.5 mM with and without royal jelly. Cells were stained with crystal violet and photographed under phase contrast microscope. mRNA levels of genes involved in intracellular antioxidant system, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GSR) were confirmed by reverse transcription-polymerase chain reaction (RT-PCR). Catalase

**Corresponding author: Email: roongs@swu.ac.th;*

activity was also determined by examining peroxidative function.
Results: Vacuole-like structures were found in the cytoplasm of HUVECs exposed to 5 mM nicotine and higher. Royal jelly alone at the concentrations lower than 2 mg/ml did not affect the structure or the survival rate of HUVECs after 1, 4, and 7 days of treatment. For cytoprotective effect, royal jelly 1-4 mg/ml mixed with 5 mM nicotine could obviously decrease the numbers of cells containing vacuole-like structures in the cytoplasm of HUVECs with the dose- and time-dependent fashion. The catalase mRNA levels and catalase activity in HUVECs exposed to 5 mM nicotine decreased significantly, but recovered when the cells were treated with royal jelly.
Conclusion: Royal jelly can be safety applied to endothelial cells even at high doses. Royal jelly is able to attenuate the abnormal vacuole-like structures induced in endothelial cell cytoplasm when exposed to nicotine. Further investigation of antioxidant gene expression showed that the mechanism possibly involves a reduction of oxidative stress via an up-regulation of catalase. Besides the supplementary food, royal jelly could be useful for endothelial cell protection from nicotine toxicity found in smoking or nicotine addiction.

Keywords: Royal jelly; protective effect; nicotine toxicity; antioxidant; catalase.

1. INTRODUCTION

Royal jelly (RJ) is a natural product in a form of a milky secretion from hypopharyngeal and mandibular glands in the head of young worker honeybees (*Apis mellifera* L.). Fresh RJ consists of water (50–65%), proteins (11–18%), carbohydrates (10–15%), fatty acids and lipids (4–8%), mineral salts (ca. 1.5%) and small amounts of polyphenols and vitamins. RJ normally kept as a lyophilized product contains 33–42% of proteins, 22–31% of carbohydrates and 15–30% of fats [1]. Volatile and extractive compounds have not been investigated. Several researchers have tried to determine the RJ composition, which is necessary for freshness and genuineness evaluation. Short-chained hydroxy fatty acids are unique for RJ, with 10-hydroxy-2-decenoic (10-HDA) and 10-hydroxydecanoic acids (10-HDDA) constituting approximately 60–80% of the total organic acids. Especially 10-HDA was mentioned to possess two important biological effects, i.e. antibiotic and antitumoural action. All larvae are fed with RJ in different duration. Brood of workers and drones are fed only first three days of life. Only queen larvae consume RJ for their entire life and become queen bees ultimately. Many advantages can be seen from queen bees, full growth with sexually active organs, double in size compared to that of worker bees and a particularly long lifespan of approximately 5-6 years while that of worker bees is only 35-40 days [2-3]. These are the reason why RJ has been claimed for health benefit, used in folk medicine for treatment of several disorders, and consumed as supplementary food.

Protective effect of RJ has attracted scientific interest resulting in several papers published in last few years. Antioxidant activity is often mentioned as the mechanism generating the protective benefit of RJ. Hepatoprotective and antioxidant activities were found in Sprague-Dawley rat with acute liver damage induced by carbontetrachloride. Lipid peroxidation monitored by malondialdehyde (MDA) and liver enzymes is reduced while the antioxidant defense systems improve [4]. Nephrotoxicity, testes damage and oxidative stress induced by cisplatin in Wistar albino rats is also improved, especially pre-treatment with RJ shows to be more effective [5-7]. RJ and green tea can exhibit the same degree of antioxidant activity to protect and restore the neprotoxicity induced by cisplatin [8]. RJ also showed dominant protective role against cadmium-induced oxidative damage and genotoxicity due to the

antioxidant activity, i.e. inhibited MDA production, and improved antioxidative status of reduced glutathione [9]. Protective action by the inhibition of lipid peroxidation was also found against genotoxicity and lipid peroxidation in plant, *Allium cepa* L. root-tip cells, caused by petroleum wastewater [10]. Protective effect of RJ against mycotoxin, fumonisin, toxicity can be found in dose-dependent manner [11].

Nicotine, an alkaloid found in tobacco, has implications in many types of tumors, especially lung cancer [12]. Nicotine is classified as one of cancer causing agents or tumor promoter probably due to angiogenic induction [13]. Several studies have recently focused on its mechanism of angiogenesis possibly through alpha 7 subunit of nicotinic acetylcholine receptor expressed on neuronal and non-neuronal cells, especially endothelial cells [14-15]. There are high therapeutic potentials in a strategy using α7nAChR agonists and antagonists for angiogenesis related diseases, especially development of novel anticancer therapies [16-17]. Nicotine induces vacuolization in cytoplasm of endothelial cells which might play an important role in lumen formation of new vessel in angiogenesis [18]. Our previous data showed vacuole-like structure found in cytoplasm of endothelial cells exposed to nicotine [19]. Inhibition of vacuolization in the cytoplasm of endothelial cells might lead to the new strategy for cancer therapy based on targeting tumor angiogenesis. Inhibiting antiangiogenesis reduces drug side effects and drug-induced resistance during cancer chemotherapy [20].

This study aims to explore the cytoprotective action of water crude extract of RJ to protect vacuole-like structure formation in endothelial cell exposed to nicotine. The capacity to inhibit vacuolization makes RJ a potential source of angiostatic agents to be developed in the future.

2. MATERIALS AND METHODS

2.1 Royal Jelly Preparation

Lyophilized RJ was purchased from a reliable company in Bangkok, Thailand. It was dissolved in the culture media, adjusted at physiological pH of 7.2 and sterilized by filtering through 0.22 μM membranes. Clear culture media was obtained and used for the endothelial cell treatment.

2.2 Cell Culture

HUVECs were a generous gift from The Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Endothelial cells were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), heparin (16 U/ml), endothelial cell growth supplement (75 μg/ml) and glutamine (2 mM). Cells were incubated at 37ºC in a humidified air with 5% CO_2 condition and subcultured by trypsinization with 0.05% trypsin-0.02% EDTA until 90% confluent monolayers were reached. All chemicals for cell culture were purchased from Gibco BRL.

2.3 Cytotoxic Assay

Colorimetric assay, that was based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by succinate dehydrogenase in mitochondria, was used

to detect the proliferative capability of HUVECs as previously described [21]. HUVECs were plated into 24-well tissue culture plate in a density of 3-4 x 10^4 cells/well with a final volume of 1 ml. After 90% confluency, the cells were treated with varying doses of RJ, 0-5 mg/ml, in complete media for 1, 4 and 7 days. The culture medium was replaced by fresh medium containing MTT dye 0.5 mg/ml and the cells were further incubated for 4 hr at 37ºC until the formation of purple formazan crystals was completed. The deep blue crystals of formazan were solubilized with acidified isopropanol and the absorbance was quantified by spectrophotometry at 570 nm (Bio-Rad 550). Percent survival was calculated against the control (untreated) group. Each sample was assayed in triplicate. All chemicals for cell viability assay were obtained from Sigma.

2.4 Cytoprotective Assay

HUVECs were seeded in 24-well culture plate and cultured as described in cytotoxic assay. After 90% confluent, the medium was replaced by fresh medium containing nicotine 5 mM with or without various concentrations of RJ (0-4 mg/ml). After, 24, 48 and 72 hr, cells were stained by 0.5% crystal violet (Merck) and the vacuole-like structures were observed and vacuole-containing cells were counted under a phase contrast microscope (Olympus BX550). Each sample was assayed in triplicate.

2.5 RNA Extraction and RT-PCR

Total RNA was isolated from treated and non-treated HUVECs using Trizol reagent (Invitrogen, USA). The purity and concentration of the isolated RNA were assessed by measuring the absorption at 260 and 280 nm. Same amounts of RNA from each sample were mixed in a 20 µl reaction mixture and single-stranded complementary DNA (cDNA) was synthesized by SuperScript RT kit (Invitrogen) according to the manufacturer’s instructions.

The following primers were used to amplify the target genes using i-Taq kit (iNtRON Biotechnology): SOD: 5′-GAGACTTGGGCAATGTGACTG-3′, 5′-TTACACCACAAGCCAAACGA-3′, 201 bp; CAT: 5′-AGTTCGGTTCTCCACTGTTGC-3′, 5′-CTTGGGTCGAAGGCTATCTGT-3′, 681 bp; GSR: 5′-CTTGCGTGAATGTTGGATGT-3′, 5′-GACCTCTATTGTGGGCTTGG-3′, 257 bp; GPX: 5′-GCCGTGCTGATTGAGAATG-3′, 5′-AGGTAGGCGAAGACAGGATG-3′, 269 bp; β-actin: 5′-AGAGCTACGAGCTGCCTGAC-3′, 5′-ACATTGTGAACTTTGGGGGA-3′, 622 bp. Amplification products obtained by PCR (Bio-Rad C1000) were electrophoretically separated on a 2% agarose gel, and stained by 2% ethidium bromide. Bands were photographed, quantitated by densitometry scanning using GeneTools analysis software (Syngene G:BOX), normalized to the mRNA level of the housekeeping gene, beta-actin and expressed as percentages with respect to control. Each sample was assayed in triplicate.

2.6 Catalase Activity Assay

Catalase activity was determined using the ability of catalase to decompose H_2O_2 molecules into water and oxygen as previously described [22]. The reaction kinetics of catalase activity was conducted at 25ºC using 50 mM phosphate buffer of pH 7.0 containing H_2O_2 as a substrate. After the 1-min incubation with cell lysates, the decrease of absorbance at 240 nm was monitored as the catalase activity to decompose hydrogen peroxide molecules. A molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$ for H_2O_2 was used. The results are expressed in µmole per µg protein per min and calculated as percentage to compare them with control.

2.7 Statistical Analysis

All data were expressed as the mean of three independent experiments ± standard deviation of control. Determination of statistical significance was performed by analysis of variance using SPSS. Statistical significance was considered when $p < 0.05$. Statistical comparisons between groups were performed using the Student's t-test.

3. RESULTS AND DISCUSSION

3.1 Effect of RJ to HUVEC Viability

Effect of RJ to HUVEC viability was performed by using MTT dye to differentiate the viable and dead cells. Inhibitory effect of RJ alone for 1, 4 and 7 days to HUVECs viability is shown in Table 1. RJ concentrations that started to decrease cell survival were 4, 3, and 2 mg/ml when exposed for 1, 4 and 7 days, respectively.

It was found that the longer the exposure to RJ, the lower the concentration that reduce cell survival. Table 1 shows response of HUVECs to RJ exposure was dose- and time-dependent manner. Toxic dose of RJ to HUVECs after a week exposure was higher than 2 mg/ml. It can be defined that RJ is quite safe for endothelial cells with the high toxic dose. In all the subsequent experiments, a maximal concentration at 5 mg/ml for 72 hr exposure time was used to see the high dose profile, while a concentration lower than 2 mg/ml was considered for cytoprotective effect and gene expression.

Table 1. Effect of RJ to HUVEC viability. Endothelial cells were treated with RJ at the concentration of 0-5 mg/ml for 1, 4, and 7 days

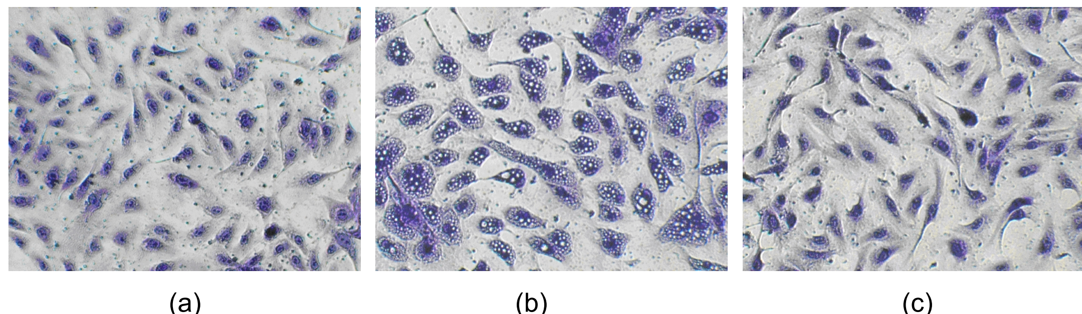
[lyophilized RJ] (mg/ml)	Percentage of survived endothelial cells		
	1 day	4 days	7 days
0.0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.1	99.52 ± 4.50	100.73 ± 4.14	100.68 ± 3.25
0.5	98.01 ± 4.53	97.98 ± 3.96	99.38 ± 3.31
1.0	99.14 ± 3.33	100.54 ± 3.22	99.68 ± 3.68
2.0	99.04 ± 3.60	99.70 ± 3.08	99.18 ± 4.14
3.0	98.75 ± 3.50	99.55 ± 3.40	95.01 ± 3.88*
4.0	99.81 ± 3.56	96.54 ± 2.71*	86.82 ± 2.63*
5.0	96.00 ± 2.08*	89.67 ± 3.45*	78.69 ± 2.40*

*Significant from normal control, * $p < 0.05$*

3.2 Cytoprotective Effect of RJ

Effect of nicotine and RJ to HUVEC viability was performed by proliferation assay as previous experiment, except that crystal violet dye was used to stain cell instead of MTT. Vacuole-like structures in endothelial cytoplasm appear in living cells and cannot be observed and counted by MTT assay. Staining with crystal violet can enhance the visualization of cell component and vacuole-like features under the microscope. Data in Fig. 1 and Table 2 shows that 5 mM nicotine caused cytoplasmic vacuole-like structure in all HUVECs. If RJ was added with 5 mM nicotine, the abnormal structure will be reduced in dose- and time-dependent pattern. Minimal RJ concentration can make HUVECs 100% recovery was 2, 2 and 1 mg/ml for the exposure of 24, 48, and 72 hr, respectively. For RJ

concentration 4 and 5 mg/ml, all HUVECs did recover, except for an unhealthy cell morphology showing a thinner than normal appearance. Cell proliferation was reduced approximately to 50% when nicotine 5 mM and RJ 4 or 5 mg/ml mixed in culture media.



(a) (b) (c)

Fig. 1. Cytoprotective effect of RJ to reduce vacuole-like structure of HUVECS exposed to nicotine 5 mM (40x).

a) Untreated endothelial cells; b) HUVECs were treated with nicotine 5 mM for 72 hr with the unusual structure in cytoplasm, vacuole-like structure; c) HUVECs were treated with nicotine 5 mM and RJ 0.5 mg/ml for 72 hr. No vacuole-like structure was observed.

In our previous paper [19], it was found that nicotine concentration at 5 mM was the lowest concentration causing vacuole-like structure with no significant inhibition of cell growth. Nicotine concentration higher than 5 mM can significantly block cell proliferation and caused vacuole-like structures in the cytoplasm of living cells in dose- and time-dependent manner. It was for this reason that we selected a nicotine concentration of 5 mM for this study.

Table 2. Effect of RJ to vacuole-like structure in HUVEC cytoplasm. Endothelial cells were treated with nicotine 5 mM and RJ at the concentration of 0, 0.1, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/ml for 24, 48 and 72 hr.

[nicotine] (mM)	[lyophilized RJ] (mg/ml)	percentage of normal endothelial cells (no vacuole-like structure in cytoplasm)		
		24 hr	48 hr	72 hr
0	0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
5	0.0	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
5	0.1	57.6 ± 7.6*	48.7 ± 11.5*	83.8 ± 9.0*
5	1.0	99.6 ± 0.5*	63.6 ± 15.9*	100.0 ± 0.0
5	2.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
5	3.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
5	4.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
5	5.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

*Significant from normal control, * $p < 0.05$*

When exposure time was extended from 24 to 72 hr, the RJ concentration needed to repair cells decreased from 2 to 1 mg/ml. RJ at 100 µg/ml seemed to be fine for vacuole-like structure formation with more than 80% (83.8 ± 9.0) cell recovery. Longer exposure should decrease RJ required concentration to a value lower than 100 µg/ml.

Endothelial cells in the body are consistently exposed to blood content, implying that a lower RJ dose would be needed to restore endothelial cells back from nicotine toxicity when RJ is administered orally. If we could close up to observe the starting point of vacuole-like structure formation, it might be possible to use nicotine at concentrations lower than 5 mM and the inhibition at this point would require RJ concentrations lower than 1 mg/ml.

Blood nicotine concentrations in individual smokers vary from 25 to 444 nmol/l [23]. The concentrations at 0.01-10 mM were found in the saliva of tobacco chewers [24]. This study used quite a high concentration of nicotine induced vacuolization (5 mM) due to the viability of HUVECs for *in vitro* condition. Previously, we found that it was necessary to use 10% FBS for HUVECs grown in culture plate for more than 10-24 hr. In this study, high concentration FBS made HUVECs healthier and hence it was necessary to use a high concentration of nicotine, 5 mM to produce vacuolization. There is no previous report mentioning vacuolization *in vivo*.

3.3 Effect of RJ on the Expression of Antioxidant Genes

RT-PCR was used to determine antioxidant gene expression for interpretation of the RJ mechanism in reducing vacuole-like structure. Expression of key genes regulating the intracellular antioxidant capacity, SOD, CAT, GPX and GSR at 24, 48 and 72 hr were analysed and normalized to the mRNA level of the housekeeping gene, β-actin. No significant difference of CAT, SOD, GPX, or GSR expression was found when the exposure to nicotine alone or in combination with RJ at 24 and 48 hr. If the exposure to nicotine alone was extended to 72 hr, down regulation of catalase expression was observed (Fig. 2). It can be seen that RJ at the concentration of 1, 2, 3 and 4 mg/ml for 72 hr was able to bring catalase expression back to normal level.

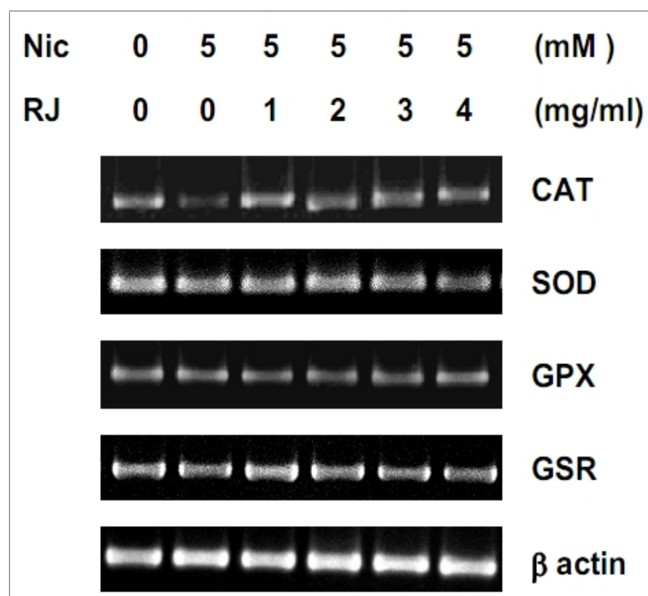


Fig. 2. Effect of RJ on nicotine-mediated expression of antioxidant genes in HUVECs. Endothelial cells were treated with 5 mM nicotine alone or in combination with 1, 2, 3 and 4 mg/ml for 72 hr.

3.4 Effect of RJ on the Catalase Activity

From RT-PCR results, RJ at the concentration of 1, 2, 3 and 4 mg/ml for 72 hr was able to bring catalase expression back to normal level. Our laboratory found the same profile of catalase expression and catalase activity. Catalase activity was reduced in HUVECs exposed to nicotine 5 mM, and RJ at the concentration of 1, 2 and 3 mg/ml for 72 hr was able to bring catalase activity back to normal level (Fig. 3).

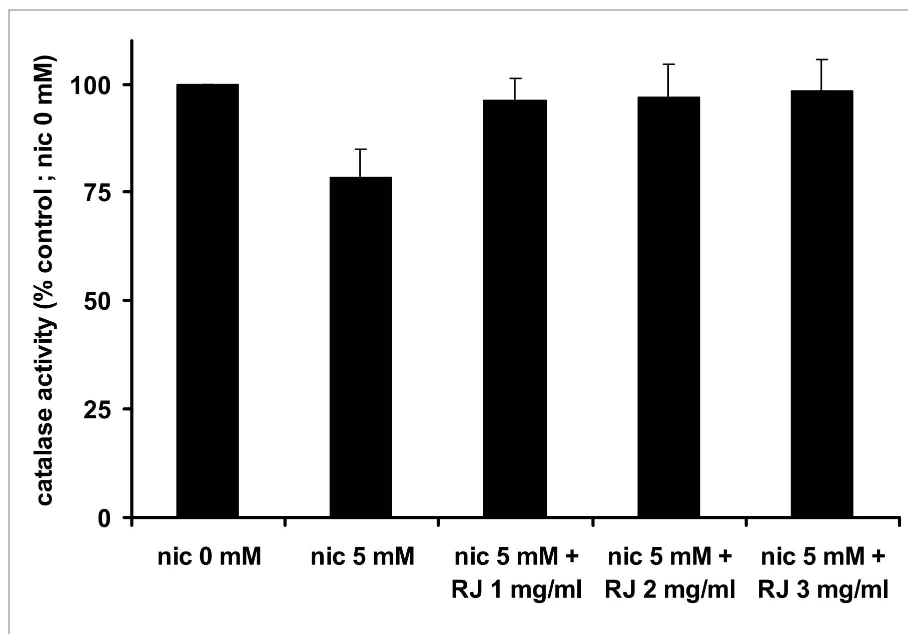


Fig. 3. Effect of RJ to catalase activity in endothelial cells treated with 5 mM nicotine and RJ at the concentration of 0, 1, 2 and 3mg/ml for 72 hr.

These RJ concentrations might look high for a unit of mg/ml. This is because the weight based on freeze-dried weight contains both water soluble and insoluble content. Our laboratory found that the part of water crude extract is 50% w/w of lyophilized RJ mass (data not shown). All experiments were performed by using lyophilized RJ dissolved in the culture media at a concentration of 0-4 mg/ml which is equivalent to a crude water extract 0-2 mg/ml. The active RJ content in all experiments was water soluble. We have to keep in mind that it is a crude extract, not a pure compound or substance. In the future, if we are able to find a compound working as active ingredient from crude water extract, the concentration should be decreased possible to the range of microgram or nanogram per milliliter. However it is better first to find that RJ in the concentration safe for cell proliferation can act against nicotine toxicity and show the antioxidant effect through restoration of the catalase gene and catalase activity.

RJ elicited a significant protective effect against oxidative stress in liver, kidney, and testes, induced by some chemicals, such as cisplatin, carbontetrachloride, resulting in reduction of lipid peroxidation and improved endogenous antioxidant defense systems, i.e. CAT, SOD, GPX and GSR [4-6]. RJ might possible exert an antioxidant to some cells other than

endothelial cells. Expression of those four genes modulating intracellular antioxidant enzymes in HUVECs exposed to nicotine were explored, but only an effect on catalase gene was found. Normally, cells use catalase to rapidly catalyze the decomposition of hydrogen peroxide which is a harmful by-product of several metabolic processes. Less reactive gaseous oxygen and water molecules are the product of the catalase reaction. This might imply that hydrogen peroxide production during vacuole-like structure formation in nicotine toxicity, with RJ then counteracting the nicotine effect via catalase activity.

A major fatty acid from RJ, 10-HDA, was found to inhibit VEGF-induced angiogenesis in HUVECs [2]. This can be explained the angiostatic agent in lipid soluble content of RJ. Our results showed that the water soluble content in RJ is able to inhibit vacuolization in endothelial cells suggesting a potential to inhibit angiogenesis. Combination of these might be the evidence expressed the possibility of both water and lipid soluble content in RJ for antiangiogenic consideration. The previous reports and our findings reveal the medical benefit of RJ protective effect to endothelial cells and to cancer therapy possibly via antiangiogenesis.

4. CONCLUSION

Our results firstly assure the safety of RJ to endothelial cells with quite high toxic dose. The more interesting is the ability of RJ to attenuate the abnormal vacuole-like structures in endothelial cells induced by nicotine. The investigation of antioxidant gene expression showed the mechanism possibly due to the reduction of oxidative stress via an up-regulation of catalase. RJ could be applied for endothelial cell protection from nicotine toxicity or some other complication in many diseases. This might be possible to add RJ in smoking cessation program due to the benefit of endothelial cell protection.

ACKNOWLEDGEMENTS

This research was supported from two sources, the annual fiscal budget of Faculty of Medicine, Srinakharinwirot University, and Srinakharinwirot University.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Han B, Li C, Zhang L, Fang Y, Feng M, Li J. Novel royal jelly proteins identified by gel-based and gel-free proteomics. J Agric Food Chem. 2011;59:10346–55.
2. Izuta H, Chikaraishi Y, Shimazawa M, Mishima S, Hara, H. 10-Hydroxy-2-decenoic acid, a major fatty acid from royal jelly, inhibits VEGF-induced angiogenesis in human umbilical vein endothelial cells. eCAM. 2009;6:489–94.
3. Isidorov VA, Bakier S, Grzech I. Gas chromatographic-mass spectrometric investigation of volatile and extractable compounds of crude royal jelly. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;885-886:109-16.
4. Cemek M, Aymelek F, Büyükokuroğlu ME, Karaca T, Büyükben A, Yilmaz F. Protective potential of royal jelly against carbon tetrachloride induced-toxicity and changes in the serum sialic acid levels. Food Chem Toxicol. 2010;48:2827-32.

5. Silici S, Ekmekcioglu O, Eraslan G, Demirtas A. Antioxidative effect of royal jelly in cisplatin-induced testes damage. Urology. 2009;74:545-51.
6. Karadeniz A, Simsek N, Karakus E, Yildirim S, Kara A, Can I, et al. Royal jelly modulates oxidative stress and apoptosis in liver and kidneys of rats treated with cisplatin. Oxid Med Cell Longev 2011; doi:10.1155/2011/981793.
7. Silici S, Ekmekcioglu O, Kanbur M, Deniz K. The protective effect of royal jelly against cisplatin-induced renal oxidative stress in rats. World J Urol. 2011;29:127-32.
8. Yapar K, Cavuşoğlu K, Oruç E, Yalçin E. Protective effect of royal jelly and green tea extracts effect against cisplatin-induced nephrotoxicity in mice: a comparative study. J Med Food. 2009;12:1136-42.
9. Cavuşoğlu K, Yapar K, Yalçin E. Royal jelly (honey bee) is a potential antioxidant against cadmium-induced genotoxicity and oxidative stress in albino mice. J Med Food. 2009;12:1286-92.
10. Türkmen Z, Cavuşoğlu K, Cavuşoğlu K, Yapar K, Yalçin E. Protective role of royal jelly (honeybee) on genotoxicity and lipid peroxidation, induced by petroleum wastewater, in *Allium cepa* L. root tips. Environ Technol. 2009;30:1205-14.
11. El-Nekeetya AA, El-Kholyb W, Abbasc NF, Ebaidb A, Amraa HA, Abdel-Wahhaba MA. Efficacy of royal jelly against the oxidative stress of fumonisin in rats. Toxicon. 2007;50:256-69.
12. Koukourakis G, Zacharias G. Nicotine has implications in different tumors types. Expert's eye making a literature analysis. J BUON. 2011;16:210-4.
13. Cardinale A, Nastrucci C, Cesario A, Russo P. Nicotine: specific role in angiogenesis, proliferation and apoptosis. Crit Rev Toxicol. 2012;42:68-89.
14. Improgo MR, Scofield MD, Tapper AR, Gardner PD. The nicotinic acetylcholine receptor CHRNA5/A3/B4 gene cluster: dual role in nicotine addiction and lung cancer. Prog Neurobiol. 2010;92:212-26.
15. Improgo MR, Tapper AR, Gardner PD. Nicotinic acetylcholine receptor-mediated mechanisms in lung cancer. Biochem Pharmacol. 2011;82:1015-21.
16. Dasgupta P, Chellappan SP. Nicotine-mediated cell proliferation and angiogenesis: new twists to an old story. Cell Cycle. 2006;5:2324-8.
17. Pillai S, Chellappan S. α7 nicotinic acetylcholine receptor subunit in angiogenesis and epithelial to mesenchymal transition. Curr Drug Targets. 2012; *in press*.
18. Park YJ, Lee T, Ha J, Jung IM, Chung JK, Kim SJ. Effect of nicotine on human umbilical vein endothelial cells (HUVECs) migration and angiogenesis. Vas Pharmacol. 2008;49:32–36.
19. Supabphol R, Supabphol A, Wattanachaiyingcharoen R, Kamkaen N. Cytoprotective effect of *Vernonia cinerea* Less. extract to human umbilical vein endothelial cells against nicotine toxicity via antioxidant activity. 2013; (*In press)*.
20. Jeong SJ, Koh W, Lee EO, Lee HJ, Lee HJ, Bae H, et al. Antiangiogenic phytochemicals and medicinal herbs. Phytother Res. 2011;25:1-10.
21. Supabphol A, Muangman V, Chavasiri W, Supabphol R, Gritsanapan W. N-Acetylcysteine inhibits proliferation, adhesion, migration and invasion of human bladder cancer cells. J Med Assoc Thai. 2009;92:1171-7.
22. Tangjitjaroenkun J, Supabphol R, Chavasiri W. Antioxidant effect of *Zanthoxylum limonella* Alston. J Med Plants Res. 2012;6:1407-14.
23. Russell MAH, Jarvis M, Iyer R, Feyerabend C. Relation of nicotine yield of cigarettes to blood nicotine concentrations in smokers. Br Med J. 1980;280:973-6.

24. Gemenetzidis E, Bose A, Riaz AM, Chaplin T, Young BD, Ali M, et al. FOXM1 upregulation is an early event in human squamous cell carcinoma and it is enhanced by nicotine during malignant transformation. PLoS One. 2009;4:e4849.

© 2013 Supabphol and Supabphol; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=159&id=13&aid=775.