The *In Vitro* Effect of Royal Jelly, Apis mellifera, on Proliferation of Human Gingival, Periodontal Ligament Fibroblasts and Human Bone Cells

นิพนธ์ต้นฉบับ

Original Article

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วารสารไทยเภสัชศาสตร์และวิทยาการสุขภาพ 2554;6(3):182-187

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บทคัดย่อ

วัตถุประสงค์: เพื่อทดสอบสารสกัดจากนมผึ้งต่อการเพิ่มจำนวนเซลล์และผลต่อการสร้างเซลล์กระดูกในเซลล์ 3 ชนิด คือ เซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกมนุษย์ (HGF) จากเอ็นยึดปริทันต์ (HPDL) และเซลล์กระดูกสะโพกมนุษย์ (HIP) **วิธีการศึกษา:** ทดสอบผลของสารสกัดนมผึ้ง (ความเข้มข้น 0.1, 0.5, 1, 5 และ 10 มก./มล.) ต่อการมีชีวิตของเซลล์ด้วยวิธี (3-(4,5 ไดเม็ทธิลไทอะซอล-2-อิล)-2,5-ไดเฟนิลเตตระโซเลียมโบรไมด์ (MTT) ศึกษาการเพิ่มจำนวนเซลล์ในช่วง 6 วันด้วยวิธีซัลโฟโรดามินบี (SRB) และวัดระดับการทำงานของสารอัลคาไลน์ฟอสฟาเตส (ALP) เพื่อวัดการตอบสนองของเซลล์ทั้ง 3 ชนิดต่อสารสกัดจากนมผึ้งโดยการเพาะเลี้ยงเป็นเวลา 14 วัน **ผลการศึกษา:** สารสกัดนมผึ้งเข้มข้น 10 มก./มล. เป็นพิษต่อ HGF และ HPDL แต่ไม่พบฤทธิ์ดังกล่าวต่อ HIP สารสกัดนมผึ้งยับยั้งการเพิ่มจำนวนเซลล์ HGF และ HPDL ที่ 5 มก./มล. โดยที่ความเข้มข้นต่ำกว่านี้กระตุ้นการเพิ่มจำนวนเซลล์ดังกล่าวได้เล็กน้อย ในทางกลับกันพบว่าสารสกัดนมผึ้งทุกความเข้มข้นไม่เพิ่มจำนวนเซลล์ HIP ในเซลล์ HPDLและ HIP ที่เติมและไม่เติมสารสกัดนมผึ้งพบการว่าการทำงานของอัลคาไลน์ฟอสฟาเตสถูกกระตุ้นใน HIP ที่ความเข้มข้นของสารสกัดนมผึ้ง 5 มก./มล. โดยไม่มีผลต่อ HPDL **สรุป:** พบผลของสารสกัดจากนมผึ้งที่แตกต่างกันในด้านการกระตุ้นการเพิ่มจำนวนในเซลล์ 3 ชนิด โดยพบทิศทางที่ดีของสารสกัดจากนมผึ้งที่ให้ผลบวกต่อการเพิ่มจำนวนและการเพิ่มการทำงานของสารอัลคาไลน์ฟอสฟาเตสในเซลล์กระดูก ซึ่งส่งผลดีสำหรับความต้องการกระตุ้นให้สร้างกระดูกในการรักษาโรคปริทันต์อักเสบหรือโรคอื่น ๆ ที่พบการทำลายกระดูก

คำสำคัญ: สารสกัดจากนมผึ้ง, เซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกมนุษย์, เซลล์ไฟโบรบลาสต์จากเอ็นยึดปริทันต์, เซลล์กระดูกมนุษย์, ความเป็นพิษต่อเซลล์

Abstract

Objective: To study the *in vitro* effect of royal jelly crude extraction (RJCE) on proliferation and osteoblastic activity in 3 cell types which were human gingival fibroblasts (HGF), human periodontal ligament fibroblasts (HPDL) and human hip bone cells (HIP). **Method:** This study used 24-hour (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxic assay, sulforhodamin B (SRB) proliferation assay and alkaline phosphatase activity (ALP) assay to evaluate the responses of these three cell types to the RJCE (concentrations of 0.1, 0.5, 1, 5 and 10 mg/ml) after 14-day culture. **Results:** It was found that RJCE was cytotoxic to HGF and HPDL at 10 mg/ml, but no cytotoxicity to HIP. From a 6-day-proliferation assay, RJCE showed proliferation inhibition to HGF and HPDL at 5 mg/ml, however, slight proliferation stimulation was observed with lower doses. On the contrary, HIP showed no proliferation response to RJCE for all dosages used up to 5 mg/ml. Stimulation on alkaline phosphatase (ALP) activity for HPDL and HIP with or without RJCE after 14-day culture was found only in HIP at 5 mg/ml RJCE, and not in HPDL. **Conclusion:** this present study showed different effect of RJCE on the 3 cell lines proliferation. The positive effect on bone cell proliferation and ALP activity looks promising for bone regeneration as needed for periodontal disease as well as other bone pathology.

Keywords: royal jelly, human gingival fibroblasts, human periodontal ligament fibroblasts, human hip bone cells, cytotoxicity

Introduction

In recent years, natural products have been widely used as health promoting agents. Part of the reasons was due to its "natural origin," which to most people, seems to be safer when compared to chemical agents. Moreover, for the products made from local ingredients, their costs would be more affordable to most consumers. Royal jelly (RJ) is a product from bee industry in the northern part of Thailand. It is produced in the hypopharyngeal and mandibular gland of worker honeybees, *Apis Mellifera*, as an exclusive food for the queen bee.[3] Chemically, RJ comprises of approximately 50 - 60% water, 18% protein, 15% carbohydrate, 3 - 6% lipid, and small amount of mineral salts and vitamins.[3] Previous studies reported that RJ exhibited numerous physiological and pharmacological effects including anti-inflammatory[11,12], antihypercholesterolemic[3], estrogenic[14] and anti-allergic activities[15,20] and augmentation of wound healing.[16,21,27] However, studies on effects of RJ on oral cells are still limited.[16]

It was previously reported that RJ stimulated as well as inhibited growth of many cell types.[1,2,4,5,13,14] It was found to stimulate proliferation of human myeloid cell line[7], MCF-7 breast cancer cell line[5,14], human monocyte[19], Tn-5Ba-4

insect cells[13] and primary rat hepatocyte.[18] At the same time there were reports on inhibitory activity of RJ on macrophage-like UM cells and human myeloid cell line.[7] Moreover RJ inhibited effect of Bisphenol A simulation of MCF-7 breast cancer cell line.[4]

Periodontium refers to tissues that surround and support the teeth. It consists of gingiva, periodontal ligament, alveolar bone and cementum. Periodontal disease is an inflammatory disease that affects the periodontium. It is caused by microorganism along with an overly aggressive immune system. Periodontal disease results in destruction of periodontium and, in the end, a loss of the tooth from its socket. The ability of the periodontal cells to repair the tissue is very crucial and many attempts had been made in order to restore lost periodontium.[1] This process, however, could not be easily achieved. It requires a series of different cell types, among which gingival fibroblasts, periodontal ligament fibroblasts, osteoblasts, endothelial cells and cementoblast.

Cell proliferation is an initial step for wound healing process. In the present study, we studied the effect of royal jelly crude extract (RJCE) on proliferation of human gingival fibroblasts (HGF), human periodontal ligament fibroblasts (HPDL) and human hip bone cell (HIP). We also analyzed its effect on alkaline phosphatase activity, an enzyme associated with bone formation.[8,24,25] The findings could be beneficial for further development of an agent to promote healing of oral wound as well as the periodontium.

Materials and Methods

Cell Culture and Reagents

Human gingival and periodontal ligament fibroblasts (HGF and HPDL) were obtained from caries-free third molars extracted for orthodontic reasons at the Faculty of Dentistry, Srinakharinwirot University with the patients' informed consent. The teeth were washed with sterile phosphate buffer saline (PBS) several times. The gingival tissues were gently removed from cervical area and the periodontal tissues were scraped from middle third of the roots by sterile surgical blades. Human primary hip bone cells (HIP), under the patients' informed consent, were a kind gift from Dr. Weerachai Singhatanadgit, Faculty of Dentistry, Thammasat University. HGF and HPDL were cultivated in Dulbecco's modified eagle medium (DMEM). HIP was cultivated in α-minimum essential medium (α-MEM). All the media were supplemented with 10% fetal calf serum (10% FCS-DMEM and 10% FCS-α-MEM), 2 mM L-glutamine, 100 IU/ml penicillin G, and 100 IU/ml streptomycin. Cultures were maintained at 37 °C in 5% CO_2. The medium and all supplements were from Gibco BRL (Carlsbad, CA, USA). HGF and HPDL were prepared from 3 teeth obtained from 3 donors and cells from passage 3 - 8 were used in the experiments. This study was approved by the Research Ethic Committee, Faculty of Dentistry, Srinakharinwirot University.

RJCE Preparation

Royal jelly, *Apis mellifera,* was purchased from Supa Farm, Chiang Mai, Thailand. They were stored at -20°C until used. Throughout the study we used the same lot to avoid variations of this natural product. RJ components were extracted with sterile PBS at a stock concentration of 50 mg/ml for 1 hour on rotation wheel. RJCE was recovered by centrifugation at 15,000 RPM for 10 min. at 4 °C. Only the supernatant was filtered and kept for experiment. RJCE was freshly prepared for each experiment. The RJCE protein profile was determined by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE) and coomasie blue staining. RJCE showed a major protein band at 55 kDa (as showed in Fig. 1) and minor bands at 49, 60, and 70 kDa. At the beginning, we varied concentrations from 10 to 80 mg/ml of royal jelly and found the highest amount of protein band at 50 mg/ml. Based on this finding, we used 50 mg/ml for a stock concentration for all experiments.

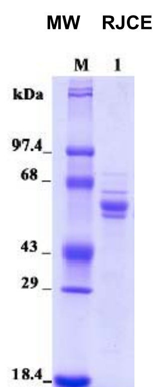


Figure 1 SDS-PAGE profile of royal jelly proteins stained with coomassie blue.

Note: MW = molecular weight marker; RJCE = royal jelly crude extraction

Another concern about royal jelly was its acidic pH. The RJCE was found to have a pH of about 3. However, buffered capacity of the culture media helped to neutralize it to pH 8 (for control, 0.1, 1 and 5 mg/ml) and to pH 7.5 at 10 mg/ml.

Cytotoxicity Test by MTT Assay

In order to find the appropriate doses of RJCE, 24-hour cytotoxicity of RJCE to HGF, HPDL and HIP was performed using MTT assay. Cells (10,000 per well) were plated in 96-well plates in 10% FCS-DMEM or 10% FCS-α-MEM overnight to allow attachment. The next day, wells were washed 3 times with media and changed to serum-free DMEM (SF-DMEM) or α-MEM (SF-α-MEM). RJCEs were treated to the cells at 0.1, 0.5, 1, 5 and 10 mg/ml (5 wells per treatment) for another 24 hours. PBS was used as a negative control. At the end of the experiment, media were changed to fresh DMEM or α-MEM (100 μl per well). Ten μL of MTT solution (5 mg/ml in PBS) was added to each well and incubated for an additional 30 min. at 37°C 5% CO_2. The colored formazan product was then dissolved in 100 μL of DMSO and optical density read at 550 nm. Mean optical density of the control group was adjusted to 100 cell percentage; consequently cell numbers of treatment conditions were also adjusted.

Proliferation Assay by SRB Method

Cells (1,000 per well) were plated in 96-well plates in 10% FCS-DMEM or 10% FCS-α-MEM overnight to allow attachment. The next day, the media were replaced with DMEM or α-MEM supplemented with 2.5% fetal calf serum (2.5% FCS-DMEM or 2.5% FCS-α-MEM). RJCEs were treated to the cells at 0.1, 0.5, 1 and 5 mg/ml (5 wells per treatment). PBS was used as a negative control. Each day one plate of each cell type was fixed with 25 ul/well of cold 50% (w/v) trichloroacetic acid (TCA, Sigma) for 1 hour at 4 °C. The conditioned media were then discarded, and plates were washed 5 times with distilled water and air-dried. After day 6, all plates were stained with 0.4% w/v sulforhodamine B solution (SRB) in 1% acetic acid (100 μl per well) (Sigma), and incubated for 10 min. at room temperature with gentle shaking. Unbound SRB was removed by washing 5 times with 1% acetic acid and the plates were then air-dried. Bound stained were solubilized with 10 mM Tris-buffered saline and the optical densities were read at 490 nm. Mean optical density of the control group was adjusted to 100 cell percentage; consequently cell numbers of treatment conditions were also adjusted.

Alkaline Phosphatase Assay

Cells (30,000 per well) were plated in 24-well plates and allow for attachment for 2 nights. The media were changed to osteogenic media which were 2.5% FCS-DMEM or α-MEM supplemented with 50 ug/ml ascorbic acid, 5 mM β-glycerophosphate and 250 nM dexamethasone in ethanol. RJCEs were treated to the cells at 0.5, 1 and 5 mg/ml (5 wells per treatment). PBS was used as a negative control. The osteogenic media and RJCE were changed every 2-3 days and the culture was maintained for a period of 14 days.

At the end of the experiment, alkaline phosphatase (ALP) activity was determined qualitatively and quantitatively. For qualitative assays, cells were washed 3 times with PBS and fixed with 70% ethanol for 10 min. The cells were then incubated for 30 min. with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate (Sigma) at 37 °C. The reaction was stopped by removing the substrate solution and washed with distilled water. ALP-positive cells were visualized by their dark blue color.

The ALP quantitative assay was modified from Pavasant et al.[27] Cells were rinsed twice with PBS and scraped in 120 μl of alkaline lysis buffer (10 mM Tris–HCl, 2 mM $MgCl_2$, 0.1% Triton-X 100, pH 10). The amount of 100 μl of each sample was incubated in the buffer containing 2 mg/ml *p*-nitrophenol phosphate (pNPP) in 0.1 M 2-amino-2-methyl-1-propanol, 2 mM $MgCl_2$, pH 10.5 at 37 °C. To stop the reaction, 0.9 ml of 0.1 M NaOH was added to each well and the absorbance was measured at 410 nm. The other 20 μl of each sample was used for protein quantitation using BCA protein assay kit (Thermo Scientific, USA) with bovine serum albumin as a standard. ALP activity was calculated as nanomolar of *p*-nitrophenol/μg protein/min and then adjusted into percentage of expression.

Statistical Analysis

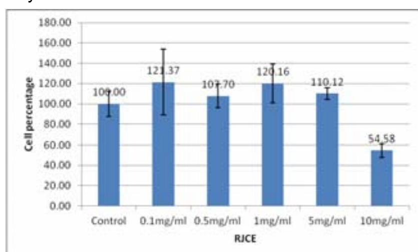
Responses among different RJCE concentrations were statistically tested using one-way analysis of variance (ANOVA) with Scheffe's test for multiple pair-wise comparisons. Statistical significance was set at $P < 0.05$.

Results

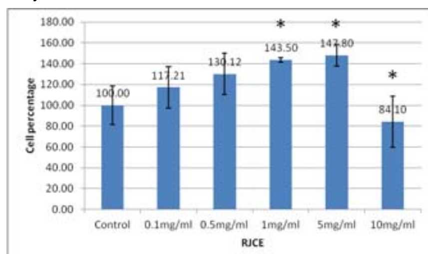
Cytotoxicity of RJCE to HGF, HPDL and HIP

Toxicity of RJCE based on MTT assay is shown in Fig. 2. The results for HGF and HPDL showed similar pattern where RJCE at 10 mg/ml was cytotoxic to both cell lines. The cell numbers dropped to 54.58% and 84.1% when compared to the controls for HGF and HPDL respectively ($P < 0.05$). On the contrary, at 0.1 – 5 mg/ml, RJCE showed a slight up-regulation of the cells numbers, which was found statistically significant at 1 and 5 mg/ml RJCE in HPDL ($P < 0.05$).

2A) Cytotoxicity of RJCE to HGF



2B) Cytotoxicity of RJCE to HPDL



2C) Cytotoxicity of RJCE to HIP

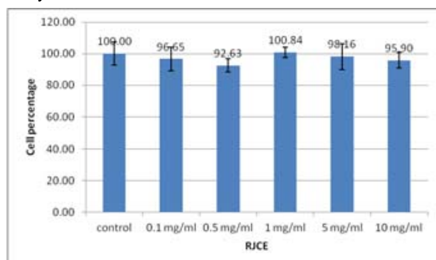


Figure 2 Twenty-four-hour cytotoxic assay of royal jelly concentrate extract (RJCE) to human gingival fibroblast (HGF) (2A), human periodontal ligament fibroblasts (HPDL) (2B) and human primary hip bone cells (HIP) (2C).

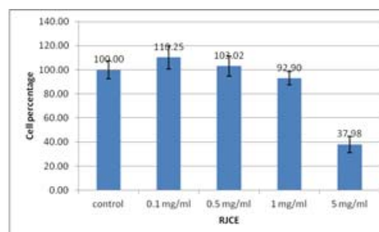
Note: Cells (10,000 per well in 96-well-plates) were plated overnight. The next day, media were changed to SF-DMEM or SF-α-MEM 100 µl per well. RJCE were treated to the cells at 0.1, 0.5, 1, 5 and 10 mg/ml (5 wells per treatment). PBS was used as a control. After 24 hour, cytotoxicity was determined using MTT assay.

For HIP cells, however, the cells seemed to be more tolerant to the RJCE treatment than the other two fibroblast lines. The HIP cell numbers in each of all treatment doses remained the same as compared to the control group (Fig. 2C). Based on these findings, we decided to use RJCE with the maximum dose of 5 mg/ml for all cell types, for the rest of the experiment.

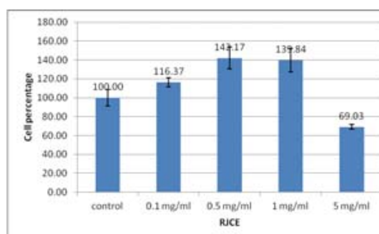
RJCE Effect on HGF, HPDL and HIP Cell Proliferation

To examine the longer effect of RJCE on the 3 cell lines, we grew the cells for up to 6 days in the presence of 0, 0.1, 0.5, 1 and 5 mg/ml of RJCE and performed SRB-proliferation assay. As shown in Figure 3 for day 6, the results of the 3 lines were in agreement with the cytotoxic assay. For HGF and HPDL, RJCE at 5 mg/ml significantly inhibited cell proliferation (*p*<0.05) whereas for the lower doses (0.1, 0.5 and 1 mg/ml) of RJCE, slight proliferation stimulation was observed. The HIP line, again, showed no proliferation inhibition by RJCE. Besides, slight proliferation stimulation was seen.

3A) HGF



3B) HPDL



3C) HIP

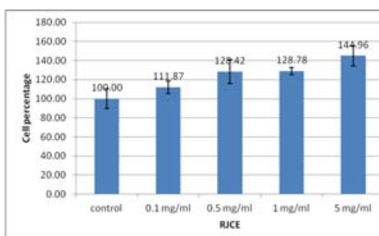


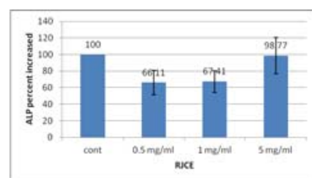
Figure 3 Proliferation assay of RJCE to HGF (3A), HPDLF (3B) and HIP (3C).

Note: Cells (1,000 per well in 96-well-plate) were plated overnight. The next day, the media were replaced with 2.5%-FCS DMEM or 2.5%-α-MEM. RJCE were treated at 0.1, 0.5, 1 and 5 mg/ml (5 wells per treatment). PBS was used as a negative control. Each day, plate was fixed with 50% TCA. At the end of the experiment, plates were stained with 0.4% w/v SRB in 1% acetic acid. Bound stained were solubilized with 10 mM Tris-buffered saline and the OD read at 490 nm. Shown here are the results of day 6.

Alkaline Phosphatase Assay of RJCE Treated HPDL and HIP

As previously reported[9] HPDL has the osteogenic potencial and the ability to produce osteogenic marker such as alkaline phosphatase enzyme when stimulated with osteogenic media. We then treated HPDL and HIP with 0.5, 1 and 5 mg/ml RJCE along with the osteogenic media for 14 days. It was found that both lines were able to produce alkaline phosphatase as determined by dark blue staining of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate (data not shown) as well as quantitative analysis by pNPP substrate (Fig. 4). ALP quantitative assay revealed no difference for ALP production with RJCE treatment in HPDL (Fig. 4A). However, RJCE at 5 mg/ml was able to induce ALP activity up to 148.35% in HIP cell lines (Fig. 4B) ($P < 0.05$).

4A) HPDL



4B) HIP

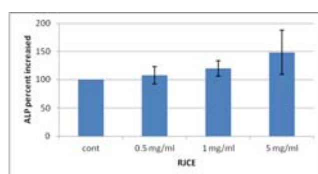


Figure 4 Alkaline phosphatase (ALP) quantitative analysis by PNPP substrate: HPDL (4A), and HIP (4B).

Discussions and Conclusion

Royal jelly (RJ) is composed of protein, carbohydrate, lipid and mineral salt.[3,22] The crude protein extract of RJ consists of both water-soluble and water-insoluble proteins. The water-soluble proteins, which we used in this study, make up 46 - 89% of the total RJ proteins.[22] As shown in Figure. 1, the protein bands between 47 - 80 kDa belong to one protein family designated as major royal jelly proteins (MRJP). To date, 5 members of this family, MRJP 1 - 5, have been identified.[22]

Periodontal ligament cells comprise of cells that potentially differentiate into various cell types including fibroblasts, osteoblasts and cementoblasts.[9] *In vitro* study found the ability of HPDL cells to differentiate to hard tissue forming cell once cultured under appropriate environment.[9] When compared to human HGF, HPDL exhibited characteristics significantly distinct by producing more protein and collagen as well as higher expression of alkaline phosphatase.[10] The latter property was suggestive of a more “osteoblast-like” character of HPDL to HGF. Interestingly, the unique characteristics of each cell type were also reflected in differences of their response to RJCE. For both fibroblast lines, HGF and HPDL, RJCE inhibited proliferation at a high dose (5 mg/ml). For lower doses, RJCE up to 1 mg/ml was able to stimulate HPDL proliferation, whereas the stimulatory effect reclined at above 0.1 mg/ml for HGF.

Our finding of HIP bone cell as the most tolerant one to high doses of RJCE as compared to HGF and HPDL is interesting and in agreement with previous reports on positive effects of RJ on bone.[2,6] It was found that RJ stimulated proliferation of mouse osteoblast-like MC3T3-E1 cells as well as enhanced collagen production in its cultured medium.[2] RJ also prevented the development of osteoporosis in experimental rats induced by ovariectomy.[6] The stimulatory effect of RJ to bone cells as reported by others[2,6] corresponded to our finding in terms of the bone cell proliferation. In bone cells, there was no inhibitory effect of RJCE. Moreover, stimulation of ALP activity in HIP bone cell was also found. This point of view supports the positive effect of RJ to regain bone. Clinically, surgical bone graft seems to be the choice at the moment. The result from this study along with many other ongoing studies may potentially lead to novel regenerative therapies for bone tissue.[30]

Taken together, this present study showed different proliferative responses of the 3 cell lines to RJCE and an ALP stimulatory effect in HIP bone cells. Further studies of RJ effect on alveolar bone cells and cementoblasts would be required. Also, clarification of the components of RJ which possess this effect along with other behavior e.g. cell adhesion, migration, collagen production would lead to a more complete picture of the effect of RJ on cells. Considering its availability and possibly positive effect on the periodontium, RJ seems to be an interesting agent in dentistry. However, awareness should be taken as RJ consumption has been linked with allergic reaction such as contact dermatitis, asthma and even anaphylaxis.[23] Another issue to keep in mind is that, being a natural product, seasonal variations could affect its chemical composition.[28] A

thorough knowledge as well as quality manufacture is crucial for achieving an effective usage of this natural product.

Acknowledgement

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