MELLIFERA RESEARCH ARTICLE

Effects of Royal Jelly Supplementation on Growth Plate Zones and Longitudinal Growth in Young Rats

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Received: 24th October, 2019; accepted: 6th November, 2019; published: 27thDecember, 2019

A B S T R A C T

Royal jelly (RJ) is secreted by the mandibular glands of worker honeybees as an essential food for the queen bee larva. In recent years, families have often used RJ supplementation for their children's growth. We investigated the effects of RJ supplementation on the growth plate of young rats and evaluated the hormone levels such as estradiol, growth hormone (GH) and insulin like growth factor-1 (IGF-1). A total of 30 rats aged 7 days were randomly divided into two groups of 15. For 15 days, 50 mg/kg of RJ was administered once a day by gavage to RJ group. Plasma estradiol, growth hormone (GH) and IGF-I levels were measured. Mean weight and tail length changes were significantly higher in the RJ group than the control group at the end of the study ($p<0.001$ and $p=0.04$). Plasma growth hormone and estradiol levels were significantly increased in the RJ group ($p=0.03$ and $p=0.04$) and the total height of the growth plate was measured significantly higher in RJ group than the control rats ($p<0.001$). In addition, the percentage of estrogen receptor expression on the growth plate was stated as 81.3% in the proliferative zone of RJ group, and as 14.3% in the control group ($p<0.001$). Our data suggested that the administration of RJ caused longitudinal bone growth and also increased estradiol and growth hormone levels, but our findings also provided the evidence of some potential estrogenic effects of RJ on growth plate.

Keywords: Royal Jelly, growth, growth hormone, estrogenic activities, growth plate

Introduction

As growth failure is one of major causes of concern and anxiety to children, adolescents, and parents, there is interest in growth promotion during childhood and adolescence. Royal jelly (RJ) is the most commonly used product of apitherapy and is in frequent use by parents as a growth supplement for children (1–3 g daily) [1,2]. RJ is yogurt-like bee milk secreted by the hypopharyngeal and mandibular glands of

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worker honeybees as an essential food for the queen bee larva. RJ contains proteins, carbohydrates, fats, free amino acids, vitamins, and minerals [3]. It is partially soluble in water and highly acidic (pH 3.4–4.5) with a density of 1.1 g/mL [4]. 10-Hydroxy-2E-decenoic (10-HDA) acid is the most important active ingredients in RJ, and the 10-HDA content can be considered as an index for estimation of quality [5,6] (Fig. 1). Many studies have reported that RJ has some potential estrogenic effects [7,8]. This honey bee-excreted biological fluid possesses estrogen-like activity, yet the compounds mediating its estrogenic effects are largely unknown. Suzuki et al [9] and Narita et al [10] demonstrated a weak estrogenic activity of RJ that it competes with 17-beta estradiol in binding to the human estrogen receptors alfa and beta, although it is much weaker than diethylstilbestrol in terms of binding affinity.

RJ is also used extensively in commercial nutritional supplements, medical products, and cosmetics and as a growth supplementation for children in many countries. Growth of long bones occurs at the growth plate, a thin layer of cartilage that separates the epiphysis from the metaphysis. An improvement in the height is significantly associated with the bone growth in length occurs at the growth plate by endochondral ossification [11]. The growth plate is regulated by a multitude of genetic and hormonal factors, growth factors, environment, and nutrition [12]. The purpose of the present study was to investigate the effects of RJ administration on the growth plate of young rats and to evaluate the hormone levels such as estradiol, growth hormone (GH) and insulin like growth factor-1 (IGF-1).

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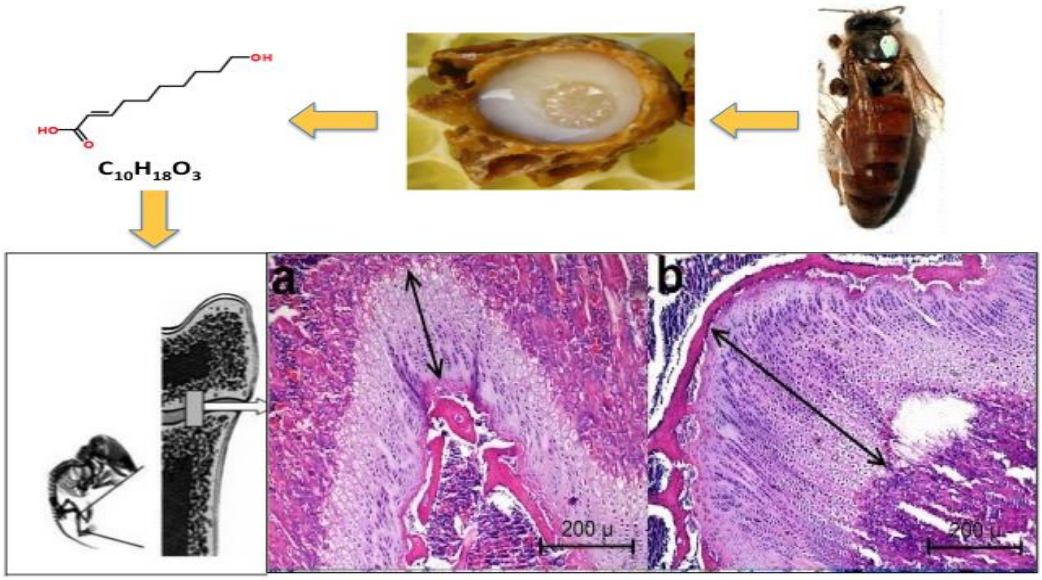

Figure 1. Chemical profile of 10-Hydroxy-2-Decenoic Acid (also called 10-HDA or royal jelly acid). It is a kind of special active substance which exists only in royal jelly in the nature.

Materials and Methods

Animals

A total of 30 Sprague-Dawley rats aged 7 days old were randomly divided into two groups each containing of 15. All the rats were breast-fed and kept in standard laboratory conditions of 22±2°C, humidity 55±5%, and a 12-hour light-dark cycle. The breastfeeding rats were not provided with food pellets during the study period but had free access to drinking water. The RJ group was administered with 50 mg/kg of RJ, once a day, by oral gavage for 15 days. The control group received 1 mL of distilled water by oral gavage once daily for 15 days. On day 0 as baseline, then weekly on Day 8, and Day 15 the animals were weighed using electronic scales accurate to ± 0.001 g (A&DGF600, Japan) and tail length was measured. The RJ was well tolerated by all the animals. There was no death in the observation period. After 15 days, the 3-week-old rats were killed and the growth plates were isolated from the proximal tibiae of each rat, thus giving a total of 30 growth plates for each group. All stages of the experiment were conducted according to the guidelines of the Institutional Ethics Committee of the S.Demirel

University (Approval number: 4062-TU2-14/03), in the line with the European Union guidelines on Animal Care.

Hormone measurements

At the end of the study, blood samples were taken from the trunk of the decapitated rats and collected into heparinized tubes at the moment of sacrifice. Plasma was separated by centrifugation at 1200× g for 15 minutes at 4°C and stored at –20°C until analysis. Plasma GH (mouse/rat rGH E023, Mediagnost, Reutlingen, Germany) and IGF-1 (mouse/rat IGF1 REF E25, Mediagnost, Reutlingen, Germany) levels were measured using ELISA kits. The minimum detection limit, and intra- and inter-assay variability for the IGF-1 ELISA kit were 90 pg/ml, 6.7%, and 6.8%, respectively. Plasma estradiol levels were measured using BioVision rat ELISA Kit (Mountain View, CA). This kit shows no species cross-reactivity. Detection range was 2–50 ng/L. These kits are highly specific and sensitive, and also have a small sample volume requirement, making them ideal for young rats.

Analysis of the growth plate

The growth plate is a highly complex, spatially polarized structure that consists of three layers: the stem cell zone, the proliferative zone and the hypertrophic zone. Histomorphometric measurements and immunohistochemical examinations were performed on the growth plate. Histomorphology of the growth plate was assessed from 5-µm sections of paraffin-embedded tissues following hematoxylin–eosin staining. The height of the growth plate was determined using the complete Olympus BX51 equipment (Olympus Optical Co. Ltd. Tokyo) consisting of a microscope connected to a computer. The images were transferred and analyzed with the Image J software. Overall growth plate height was measured by determining the central region on the long axis of the tibia. Horizontal lines were drawn along the contours of both the epiphysis on the proximal side of the growth plate and the chondro-osseous junction on the distal side of the growth plate. Growth plate zone heights were totaled to give the total growth plate height representing the distance between the primary and secondary centers of ossification. The total height of the growth plate was calculated as the average of 10–20 measurements/growth plate.

Immunohistochemical analysis

Immunohistochemical analysis for Ki-67, Estrogen receptor (ER) and IGF-1 receptor was performed on formalin-fixed, paraffin embedded tissues, using the streptavidin-biotin-peroxidase technique. The sections

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were incubated with precisely diluted mouse monoclonal antibodies against Ki-67 (Rabbit monoclonal [SP6], MA, USA), Estrogen Receptor (Rabbit polyclonal to ER alpha, MA, USA) and IGF-1 receptor (IGF-1, Rabbit polyclonal to IGF-1 Receptor, MA, USA). Positive stained cells of proliferative and hypertrophic chondrocytes per column were counted using Olympus BX51 equipment. The percentages of IGF-1 and ER-positive chondrocytes were assessed in the proliferative and hypertrophic zones of the growth plate. The Ki-67 cell proliferation index was calculated on the basis of the percentage of positive stained nuclei [13]. At least 2000 cells in the growth plate were counted in each group. Positive cells were counted as recommended by Iamaroon et al [14].

Statistical analysis

All data were presented as the means ± standard deviation (SD) for each group. The differences between the groups were evaluated using an unpaired t-test. (SPSS version 17, Chicago, IL). Differences were considered significant at a value of $p<0.05$.

Results and Discussion

The changes of weight and tail length

The changes of weight and tail length of the groups were shown in Table 1. The body weights and tail lengths were similar in both groups at baseline. Following the RJ administration, there was a marked increment in mean weight change in the RJ group compared with the control group (41.4±7.1 g *vs.* 31.5±4.8 g, $p<0.001$). The change of the tail length was significantly higher in the RJ group than in the control group (3.7±0.6 cm *vs.* 3.6 ± 0.3 cm, $p=0.04$) (Fig. 2).

Table 1. Weight and tail length changes in rats supplemented with Royal Jelly (RJ) for 15 days

		Royal Jelly	Control	p
Weight (grams)				
	Baseline	46.5 ± 6.1	48.7 ± 3.4	0.756
	8^{th} day	69.7 ± 9.2	59.9 ± 4.4	0.001
	15^{th} day	87.9 ± 11.9	80.3 ± 6.7	0.04
	Changes (1-15th days)	41.4 ± 7.1	31.5 ± 4.8	<0.001
Tail length (cm)				

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	Baseline	7.8 ± 0.5	7.5 ± 0.3	0.161
	8th day	9.8 ± 0.5	9.2 ± 0.4	0.04
	15th day	11.5 ± 0.5	11.1 ± 0.4	0.06
	Changes (1-15th days)	3.7 ± 0.6	3.6 ± 0.3	0.04

Hormonal measurements at the end of the study

Hormonal measurements at the end of the study were presented in Table 2. Compared with the control group, the GH levels were found to be higher in the RJ group following RJ administration (2.8±0.7 ng/dL *vs.* 1.05±0.6 ng/dL, p=0.03).

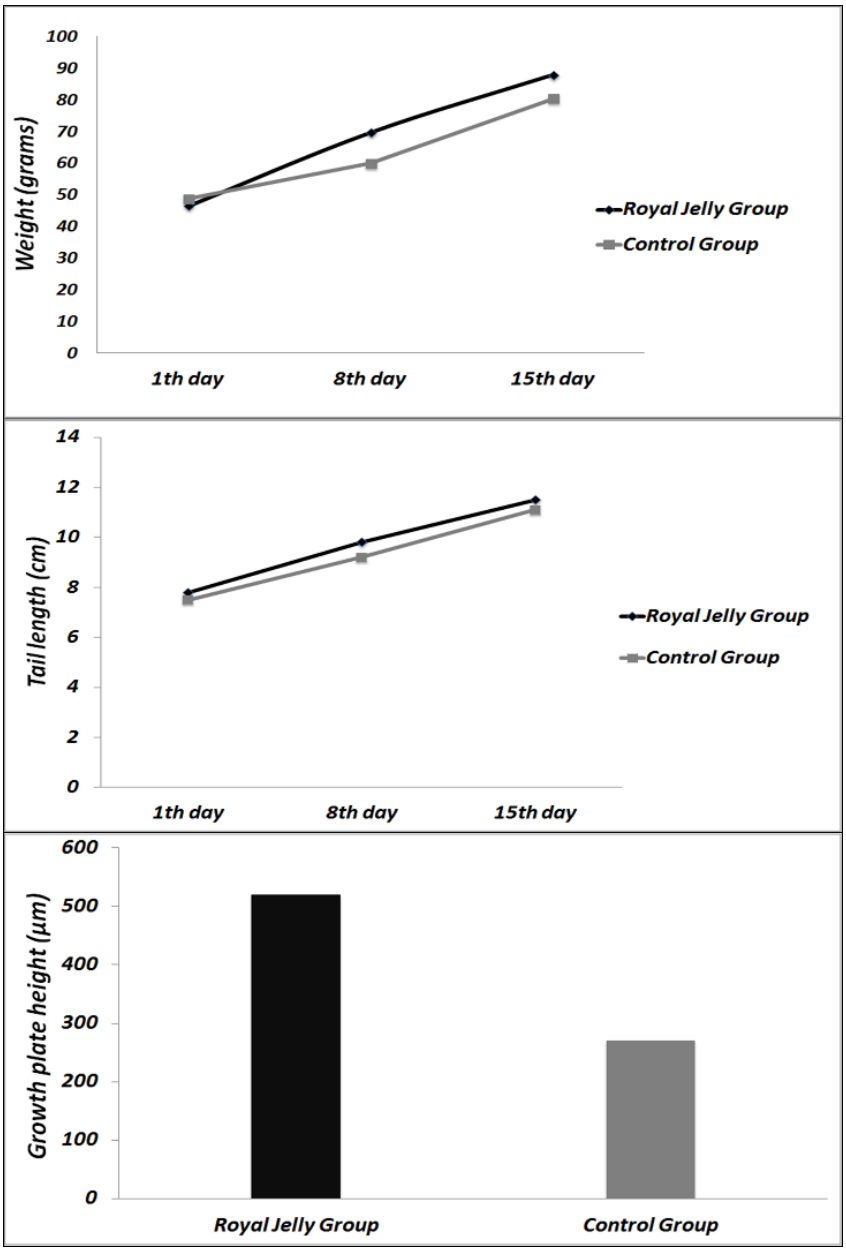

Figure 2. Mean weight, tail length measurements at baseline, 8th and 15th days. At the end of the study, tibial growth plate heights of the groups were shown.

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Table 2. Effects of Royal Jelly administration on hormone levels

	Royal Jelly	Control	p
Estradiol (pg/mL)	708 ± 53	582 ± 85	0.04
Growth hormone (ng/dl)	2.8 ± 0.7	1.05 ± 0.6	0.03
IGF-1 (ng/dL)	404 ± 4	207 ± 27	0.175

The plasma estradiol level was also higher in the RJ group (708±53 pg/mL vs. 582±85 pg/mL, p=0.04) than the controls at the end of the study. However; there was no significant difference among the groups for IGF-I levels (404±4 ng/dL *vs.* 207±27 ng/dL, p=0.175) (Fig. 3).

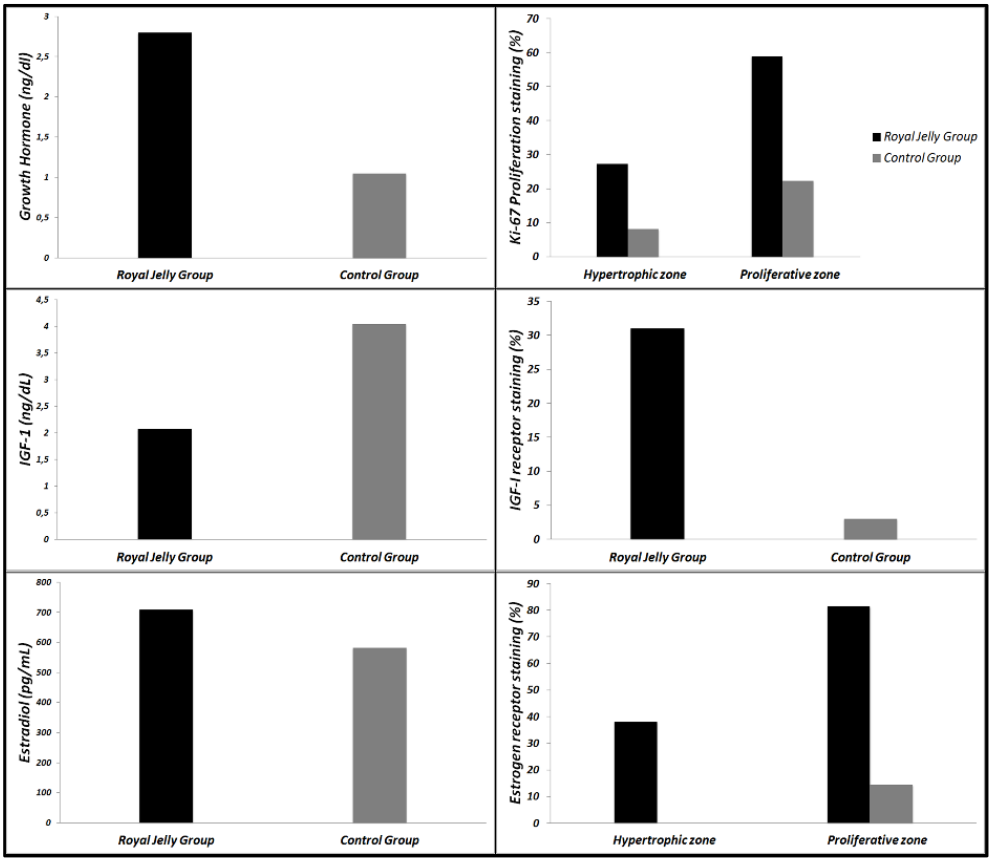

Figure 3. The effects of oral administration of Royal Jelly on hormone levels (Growth hormone, IGF-I and estradiol) and on the growth plate (immunohistochemical stainings; Ki-67 proliferation, IGF-1 receptor and estrogen receptor stainings, results are expressed as % of cell count).

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Comparisons of growth plate measurements

The tibial growth plates at the termination of the experiment on Day 15 are presented in Fig. 4. The total height of the growth plate was measured as 520±10 μm in the RJ group, and as 270±8 μm in the control group ($p<0.001$) at the end of the study (Table 3, Fig. 4a and b). The proportions were determined for the cell density (percentage of cell counts) in the proliferative and hypertrophic zones of the growth plate. Increased estrogen receptor expressions were determined in the proliferative zones of the RJ group compared to the control group (81.3±17.6% *vs.* 14.3±16.3%, $p<0.001$). The percentage of the estrogen receptor expressions in the RJ group were also significantly increased in the hypertrophic zones ($p<0.001$) (Fig. 4g and h). The IGF-1 receptor expression staining was determined as higher expression percentages in the growth plates of the RJ group than in the control group (31±14.7% *vs.* 3±5%, $p<0.001$) (Fig. 4e and f). We found that Ki-67 staining was highly expressed in the hypertrophic zones of the RJ group (27.3±7.9% *vs.* 8±4.6%, $p<0.001$) compared to the control group and there was a significant difference in the proliferative zones among the groups (59±14.9% *vs.* 22.3±17.8%, $p<0.001$) (Fig. 4c and d). In recent years, families have often used RJ supplementation for their children's growth. The present report investigated the possible effects of oral RJ administration on the longitudinal growth and the growth plate of the young rats. We demonstrated in this study that RJ might have some benefits in weight gain and the longitudinal growth, which is an effect linked to increased plasma GH and estradiol levels. Many systemic hormones regulate the longitudinal bone growth including GH, IGF-1, insulin, thyroid hormone, glucocorticoids and sex steroids [15]. In this study, we found higher plasma GH and estradiol levels in the blood samples of the RJ administered group compared to the control group at the end of the study. A study by Narita et al [10] showed that oral administration of RJ to normal female mice caused an increase in bone content and up regulation of gene expression of type I procollagen. The difference of plasma IGF-1 levels was not significant among the groups. It has been argued that IGF-1 locally produced in the growth plate is of greater importance in the regulation of growth plate cartilage than systemic levels of IGF-1 [16]. We detected that the IGF-1 receptors were expressed

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significantly higher percentage staining in the growth plate of the RJ rats than the control rats although no significant difference in IGF-1 levels was found among the groups. These data support the view that is a more local action of IGF-1 which directly targets the growth plate chondrocytes. Ki-67 is a human nuclear protein the expression of which is strictly associated with cell proliferation and which is widely used in routine pathology as a "proliferation marker" to measure the growth fraction of cells [17]. The expression of the Ki-67 positive cells (the Ki-67 labeling index) is closely associated with cell proliferation [17, 18]. Although the rate of new cell production in the proliferative zone is an important factor in bone formation, the hypertrophic zone plays a key role as well [19]. We showed increased Ki-67 expressions particularly in the proliferative zone of the growth plate of the RJ group compared to the control group growth plates (Figs. 4c and d). We found also highly expressed IGF-1 and estrogen receptors locally in the growth plate chondrocytes following RJ administration. These data suggest that the content of RJ (most probably the estrogen compounds of RJ) can promote the growth stimulation in young rats. Estrogens are important endocrine regulators of skeletal growth and maintenance in both females and males [20, 21].

In present study, RJ was seen to increase the longitudinal growth, but it also had estrogenic effects on the growth plate zones in growing rats. We detected significantly higher estradiol and GH levels in blood samples and positive estrogen receptor expressions were demonstrated on growth plate in the RJ administered rats compared to the control group (Figs. 4g and h). Both of the estrogen receptors alfa and beta are expressed by bone and growth plate cartilage of humans and other species [22, 23]. Estrogens are crucial regulators of the GH/IGF-1 axis [24, 25] and therefore, some of the effects of estrogens on skeletal growth may be indirect via the modulation of the GH/IGF-1 axis. In the current study, a significant increase was determined in the total growth plate height of the rats after 15 days of RJ supplementation, probably through increased estrogenic activity. These findings therefore strongly support the hypothesis that RJ has an estrogenic effect on growth plate chondrocytes and thereby could affect linear bone growth in children administered with orally RJ.

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Table 3. Effects of Royal Jelly administration on growth plate height and growth plate immunohistological staining in growing rats at the end of the study.

	Royal Jelly	Control	p
Growth plate total height (µm)	520 ± 10	270 ± 8	<0.001
Estrogen receptor staining (%)			
Hypertrophic zone	38 ± 20	0 ± 0	<0.001
Proliferative zone	81.3 ± 17.6	14.3 ± 16.3	<0.001
Ki-67 receptor staining (%)			
Hypertrophic zone	27.3 ± 7.9	8 ± 11.6	<0.001
Proliferative zone	59 ± 14.9	22.3 ± 17.8	<0.001
IGF-1 receptor staining (%)	31 ± 14.7	3 ± 5	<0.001

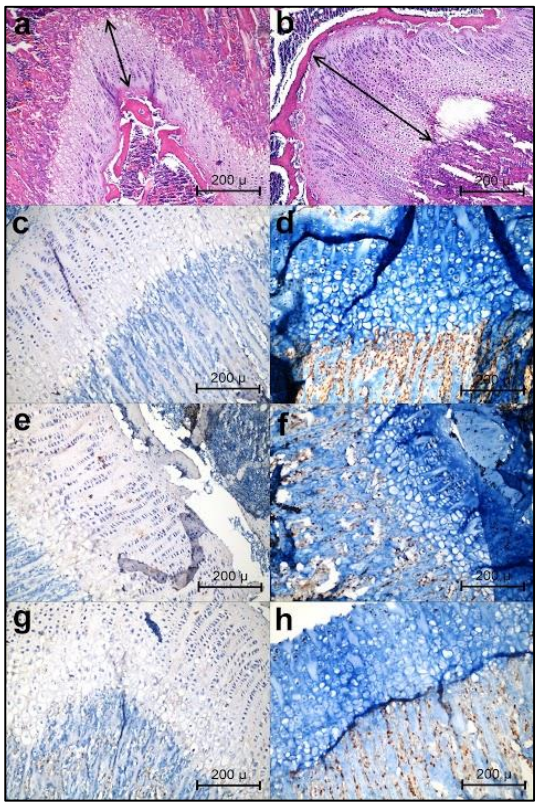

Figure 4. Total growth plate and receptor expressions of the growth plates of RJ group (b, d, f and h) and the control group (a, c, e and g). Images of growth plates from the proximal tibia at the end of the study. (Black arrows indicate the total growth plate height). A positive reaction for Ki-67 (d), IGF-1 receptors (f) and estrogen receptors (h) was observed as

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Conclusion

This study has some limitations which have to be pointed out. The small study group do not allow us to draw any conclusion about the effectiveness of this RJ supplementation on growth. Furthermore, the follow-up was limited. Larger series with long-term follow-up are needed to confirm the effectiveness of the RJ on human studies.
In conclusion, exposure of young rats to RJ by orally caused increased estradiol and growth hormone levels and also longitudinal bone growth, but our findings also provided the evidence of some potential estrogenic effects of RJ on growth plate. However, increased estrogenic activity is also important for the cessation of growth by inducing growth plate closure and this effect of RJ may diminish the final height potential. It can be said that despite the common usage of RJ by parents for children, there still seems to be much to study and learn about the effects of RJ on children.

Arı Sütünün Genç Ratların Büyümesine ve Büyüme Plağı Zonları Üzerine Etkisi

Öz: Kraliçe arıların ana besin öğesini oluşturan Arı sütü, işçi arıların mandibular bezlerinden salgılanmaktadır. Son yıllarda, ailelerin çocuklarının büyümesine yardımcı olmak için Arısütü'nü uygulamasında artış olduğu gözlenmektedir. Bu çalışmada arısütü verilen genç sıçanlarda büyüme plağı zonlarına olan etkisi ve hormonal etkilerini (östrodiol, büyüme hormone ve insulin benzeri büyüme faktörü-1) araştırdık. 30 adet 7 günlük sıçan randomize olarak iki gruba ayrıldı. Bir gruba annesütünün yanısıra 15 gün süresince gavaj yolu ile günde 50 mg/kg Arı sütü uygulandı. Çalışmanın sonunda RJ verilen grubun göre ortalama ağırlığı kontrol grubuna daha fazla ve ortalama kuyruk uzunlukları daha uzundu ($p<0.001$; $p=0.04$). Plazma büyüme hormonu ve östradiol seviyesi RJ g rubunda daha yüksek olarak sonuçlandı ($p=0.03$; $p=0.04$). Büyüme plağının uzunluğu RJ grubunda control grubuna göre daha uzun olduğu tespit edildi. Ayrıca büyüme plağının proliferative zonunda östrojen reseptör ekspresyonu RJ grubunda control grubuna göre daha fazla olduğu gözlendi ($p<0.001$). Çalışma sonunda büyüme plağı zonları, östrodiol, büyüme hormonu ve insulin benzeri büyüme faktörü-1 ölçüldü. Bu çalışma sonucunda Arı sütü'nün büyüme plağı zonlarında ve serumda östrodiol ile büyüme hormonunun artışı olduğu ancak büyüme plağının uzamasının Arısütünün potansiyel östrojenik etkisine bağlı olacağı belirtildi.

Anahtar Kelimeler: Arı sütü, büyüme, büyüme hormonu, östrojenik aktiviteler, büyüme plağı

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