

Histological aspects of rat ovaries supplemented with bee pollen from *Cocos nucifera*

Aspectos histológicos de ovários de ratas suplementadas com pólen apícola de *Cocos nucifera*

Aspectos histológicos de ovarios de ratas suplementados con polen de abeja de *Cocos nucifera*

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Abstract

Bee pollen is a food supplement for its medicinal properties. It has a high concentration of vitamins, mineral salts, proteins, phenolic compounds and carbohydrates, which are beneficial to health. The objective of this work was to evaluate the effects of bee pollen supplementation on the ovarian performance of rats. Fifty pubescent rats were separated into groups maintained with a common feeding routine. Two experimental groups of 15 animals each were supplemented by intragastric administration with 50 mg of bee pollen from *Cocos nucifera*, for 30 and 90 days. The estrous cycle was followed in the last 12 days of treatment. In the first estrus after treatment, euthanasia was performed by intraperitoneal administration of sodium thiopental (50 mg/kg), to remove the ovaries for making histological sections. The number and types of ovarian structures present were analyzed: secondary follicle, Graafian follicle, atresic follicle and corpus luteum. In animals supplemented for 30 days there was a significant increase in the number of atresic follicles compared to controls. This increase also occurred in the supplemented 90 days compared to 30 days. It was also found a decrease in the number of corpora lutea, suggesting that the effects are time dependent.

Keywords: Bee pollen; Functional food; Ovary histology.

Resumo

O pólen de abelha é um suplemento alimentar por suas propriedades medicinais. Possui alta concentração de vitaminas, sais minerais, proteínas, compostos fenólicos e carboidratos, que são benéficos à saúde. O objetivo deste trabalho foi avaliar os efeitos da suplementação de pólen de abelha no desempenho ovariano de ratas. Cinquenta ratas púberes foram separados em grupos mantidos com uma rotina alimentar comum. Dois grupos experimentais de 15 animais cada foram suplementados por administração intragástrica com 50 mg de pólen de abelha de *Cocos nucifera*, por 30 e 90 dias. O ciclo estral foi acompanhado nos últimos 12 dias de tratamento. No primeiro estro após o tratamento, foi realizada a eutanásia por administração intraperitoneal de tiopental sódico (50 mg/kg), para retirada dos ovários para realização de cortes histológicos. Foram analisados o número e os tipos de estruturas ovarianas presentes: folículo secundário, folículo de Graaf, folículo atrésico e corpo lúteo. Nos animais suplementados por 30 dias houve um aumento significativo no número de folículos atrésicos em relação aos controles. Esse aumento também ocorreu nos 90 dias suplementados em relação aos 30 dias. Também foi encontrada uma diminuição no número de corpos lúteos, sugerindo que os efeitos são dependentes do tempo.

Palavras-chave: Pólen de abelha; Alimento funcional; Histologia do ovário.

Resumen

El polen de abeja es un complemento alimenticio por sus propiedades medicinales. Tiene una alta concentración de vitaminas, minerales, proteínas, compuestos fenólicos y carboidratos, que son beneficiosos para la salud. El objetivo de este trabajo fue evaluar los efectos de la suplementación con polen de abeja en el desempeño ovárico de ratas hembra. Cinquenta ratas púberes se separaron en grupos mantenidos en una rutina de alimentación común. Dos grupos experimentales de 15 animales cada uno fueron suplementados por administración intragástrica con 50 mg de

polen de abeja Cocos nucifera durante 30 y 90 días. Se monitoreó el ciclo estral en los últimos 12 días de tratamiento. En el primer celo posterior al tratamiento se realizó la eutanasia mediante la administración intraperitoneal de tiopental sódico (50 mg/kg) para extraer los ovarios para cortes histológicos. Se analizó el número y tipos de estructuras ováricas presentes: folículo secundario, folículo de Graaf, folículo atrésico y cuerpo lúteo. En los animales suplementados durante 30 días hubo un aumento significativo del número de folículos atrésicos en relación a los controles. Este aumento también se produjo en los 90 días complementados en relación a los 30 días. También se encontró una disminución en el número de cuerpos lúteos, lo que sugiere que los efectos dependen del tiempo.

Palabras clave: Polen de abeja; Comida funcional; Histología ovárica.

1. Introduction

Pollen appears in the form of microscopic grains, contained in the male reproductive system of flowers. When collected by worker bees, it undergoes biochemical modifications, by the addition of nectar and its salivary secretions (Brasil, 2001a; Mašková et al., 2019). Insalivation promotes the addition of enzymes, amino acids and vitamins, functional elements of rich nutritional value, important in human health. It is recommended to use 1 to 2 tablespoons daily (Brasil, 2001a; Li et al., 2018). In hives, it is used as a source of protein for feeding larvae. For humans, it is said to be effective in treating anemia and prostate disorders (Komosinska-Vassev et al., 2015; Tuoheti et al., 2020).

The chemical composition of pollen is highly complex and probably not yet fully understood. Generally, it fluctuates according to seasonal variation and according to the chemical components of the plant species and the environmental conditions during its elaboration; for this reason, each type of pollen has a different amount of vitamins, proteins, carbohydrates, minerals, flavonoids and sugars (Thakur & Nanda, 2020; Tuoheti et al., 2020). It is important to use a pollen of known botanical origin for human consumption, since in a total of 60% of the bee pollen samples were identified pyrrolizidine alkaloids, suggesting that the commercial product may not be safe, if it is of origin from plants producing these compounds (Dübecke et al. 2011; Inacio et al., 2020). These substances can be hepatotoxic, pneumotoxic, genotoxic and carcinogenic to mammals (Stegelmeier et al., 1999; Kast et al., 2019). However, in general, there are rare cases of side effects and toxicity resulting from the use of bee pollen described in the literature. However, in a study by Akiyasu et al. (2010) it was reported that pollen consumed as a food supplement caused acute renal failure, respiratory distress, excessive weight gain and anuria. There are reports in the literature that the supplementation of pregnant rats with bee pollen throughout the gestational period had a great harmful effect on mothers and fetuses (AbdEl-Gawad, 2010). The evaluation of the toxic potential is important, considering that data regarding the risks of using bee pollen as a dietary supplement are still rare in the literature.

Pollen is basically composed of water, proteins, carbohydrates, lipids and minerals such as: calcium, copper, iron, magnesium, phosphorus, potassium, silicon, sulfur, aluminum, manganese, nickel, titanium and zinc. In addition, it has vitamins A, B, C, D, E, enzymes and coenzymes (Thakur & Nanda, 2020; Tuoheti et al., 2020). It contains an average of 22.7% protein, including 10.4% essential amino acids such as methionine, lysine, threonine, histidine, leucine, isoleucine, valine, phenylalanine and tryptophan (Komosinska-Vassev et al., 2015). It has significant amounts of flavonoids and polyphenolic substances, to which its therapeutic effects have been attributed (Kroyer & Hegedus, 2001; Tuoheti et al., 2020).

Daily pollen intake has been recommended, as it regulates intestinal functions, has beneficial effects on the cardiovascular system, skin, vision and has an antibiotic, antidiarrheal, anticancer and antioxidant effect. Thus, one can assess its importance as a food supplement, showing its relevance as a functional food (Kroyer & Hegedus, 2001; Silva et al., 2006; Saric et al. 2009; Silva et al., 2009; Komosinska-Vassev et al., 2015; Thakur & Nanda, 2020; Tuoheti et al., 2020). It helps to prevent osteoporosis, as it inhibits cytokines that recruit osteoclasts and opposes bone resorption (Yamagushi, 2006; Kafadar et al, 2012), it makes up for dietary deficiency, thus resulting in a functional balance (Kroyer & Hegedus, 2001; Tuoheti et al., 2020), improves the profile blood biochemistry, semen quality and fertility (Attia et al., 2011). In addition, it reduces blood cholesterol levels, improves liver function in chronic hepatitis, prevents the formation of atherosclerotic plaques in blood

vessels, and demonstrates high antioxidant, probiotic, and prebiotic potential (Komosinska-Vassev et al., 2015; Denisow & Denisow-Pietrzyk, 2016; Urcan et al. 2017; Kieliszek et al., 2018; Kafantaris et al., 2020).

Because it contains a variety of plant hormones, such as heteroauxin and animal sex hormones, such as steroids, it is assumed to be effective in the treatment of sterility and in polycystic ovary syndrome (Naseri et al., 2021). For this reason, the aim of this work was to investigate the possible effects of bee pollen from coconut floral origin on the functional dynamics of ovaries of supplemented rats. To meet the objectives of this work, the acute, subacute and chronic effects of supplementation with pollen from *Cocos nucifera* (*C. nucifera*) were investigated.

2. Methodology

2.1 Bee pollen sample

The bee pollen used in this experiment is monofloral, of coconut floral origin (*C. nucifera*), from the municipality of Aracaju-SE. A 200 g sample was manually cleaned and dehydrated in an oven (IMESUL – Metal Apícola LTDA, 20 kg) at 45° C to constant weight (160 g). It was then ground to pass through a size 30 mesh (NetLab) and stored in sealed glass vials at 5° C in a refrigerator. The sample was provided by the Center for Apicultural Studies of the University of Taubaté, Taubaté-SP, where the palynological and physical-chemical analyzes were performed.

2.2 Palynological analysis: identification of pollen rate in the sample

The sample was prepared by the acetolysis method (Erdtman, 1960) and for the quantitative analysis, 1500 samples of pollen grains were used (Vergeron, 1964). The relative and mean abundance and relative frequency of pollen grains in the samples were calculated according to Louveaux et al. (1978).

2.3 Physicochemical analysis of the sample

The physicochemical analyzes such as moisture, protein, lipids, ash and sugars were carried out using methodologies previously described in the literature: to determine the moisture content, 2.0 g of bee pollen were dried at 105° C up to constant weight to determine water loss. Ash content was determined after incineration at 560° C. The pH was measured after mixing 2.0 g of sample pollen with 5 mL of ultrapure water (18 MΩ, AKSO) (Bárbara et al, 1983). Protein content was determined by determining the % N pattern using the Kjeldahl method, with a conversion factor of 6.25 or 5.6 (Campos et al., 2008). Total sugars were determined by the dinitrosalicylic acid method (Bárbara et al, 1983). Lipid content was determined by extraction with dichloromethane using a soxhlet reflux apparatus as described by Archini et al. (2006). Total acidity was determined using 2.0 g of the sample mixed with 5.0 mL of ultrapure water and titrated with 0.1 M NaOH to pH 8.5 (Bárbara et al, 1983).

2.4 Animal model

Wistar rats (*Rattus norvegicus*) bred and maintained in the vivarium of the Faculty of Pindamonhangaba were used. Fifty nulliparous females aged 55 ± 5 days were kept in number of five per cage, in a room with controlled temperature ($24 \pm 2^\circ$ C), following a 12-hour light/dark cycle (light period of 7:00. h to 19:00 h), receiving commercial feed (Nuvilab® CR1, 2.95 Kcal/g) and water ad libitum.

The experimental protocols used in this study were approved by the Committee on Ethics in the Use of Animals of the Faculty of Pindamonhangaba, protocol CEUA 016/2011. The animals were divided into four groups: GI- Subacute toxicity: 15 females that were treated daily, intragastrically, with 50 mg of *C. nucifera* bee pollen, homogenized with double distilled water to form a solution with an almost liquid consistency. The estrous cycle of each animal was monitored daily in the last 12 days of each treatment, through analysis of the vaginal lavage under light microscopy (proestrus: characterized by nucleated basal

cells; estrus: predominance of anucleated keratinized cells; metaestrus: combination of leukocytes with keratinized cells; diestrus: intense presence of leukocytes) (Marcondes et al., 2002). The animals were euthanized after completing 30 days of treatment. GII- Chronic toxicity: 15 females who received the same treatment (50 mg) for 90 days; after, they were euthanized. GIII- Control Group: 20 females that received 1.0 mL of water as a placebo, by the same route and for the same periods. Of these animals, 10 were euthanized after completing 30 days and 10 after completing 90 days. In the first estrus at the end of the experimental period, each rat was previously weighed and then euthanized with the use of an excessive dose of sodium thiopental (50 mg/kg), via intraperitoneal.

During necropsy, the ovaries of the animals in each group were collected separately and stored in a 10% buffered formaldehyde solution (Sigma Aldrich), for the preparation of histological sections, which were performed in a commercial laboratory as routine. These histological sections were analyzed under a light microscope. The counting of growing follicles (with more than two layers of follicular cells) and antral follicles (with the presence of the antrum), atresic follicles and corpus luteum were counted (Pedersen & Peters, 1968).

2.5 Data analysis

For statistical analysis of the results, the software “BioEstat” version 5.0 was used, using the Kruskal-Wallis non-parametric analysis of variance, complemented with the Dunn or Student-Newman-Kleus test, depending on the variable analyzed. Significance was given for $p < 0.05$.

3. Results

The palynological analysis identified *Cocos nucifera* as the main taxon in the bee pollen sample (99.0%) as shown in Figure 1.

Figure 1. Photo of coconut pollen used in the present work (A). Microphotograph of coconut pollen grain (40X magnification) (B).



Fonte: Authors.

The data obtained in the physicochemical analyzes of *C. nucifera* pollen are presented in Table 1.

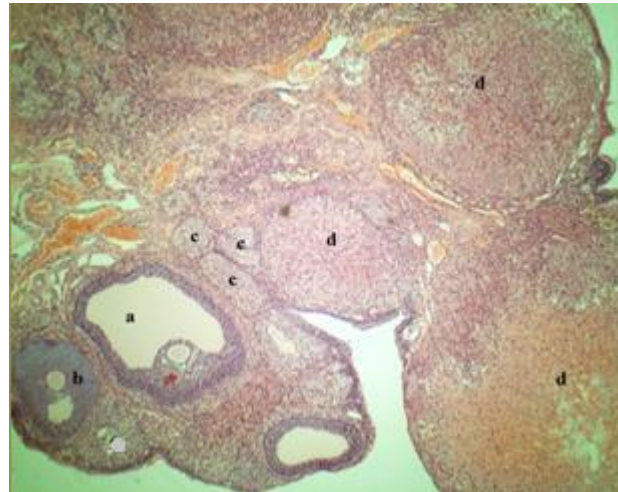
Table 1. Results of physicochemical analyzes in the dehydrated sample of bee pollen of *C. nucifera*.

Analysis	Content	Acceptable level*
Free acidity (mEq/kg)	110.00	Maximum 300 mEq/kg
Total sugars (%)	31.56	14.5 to 55% (m/m) dry base
Ashes (%)	2.54	Maximum 4% (m/m) dry base
Lipids (%)	8.35	Minimum 1,8% (m/m) dry base
Minerals (%)	3.13	Unspecified
pH	5.30	4 a 6
Proteins (%)	19.60	Minimum 8% (m/m) dry base
Moisture (%)	4.00	Maximum 4% (m/m) dry base

* Technical Regulation on the Identity and Quality of Bee Pollen (TRIQ) (Brasil, 2001b). Source: Authors

The treated animals showed no significant weight change when compared to the control group. The ovarian structures observed in the histological sections are shown in Figure 2.

Figure 2. Histological section of rat ovary supplemented with *C. nucifera* bee pollen for 90 days: a- antral follicle, b- secondary follicle, c- atresic follicles, d-corporate luteum (HE-100X).



Source: Authors.

The results regarding ovarian follicular quantification are shown in Table 2.

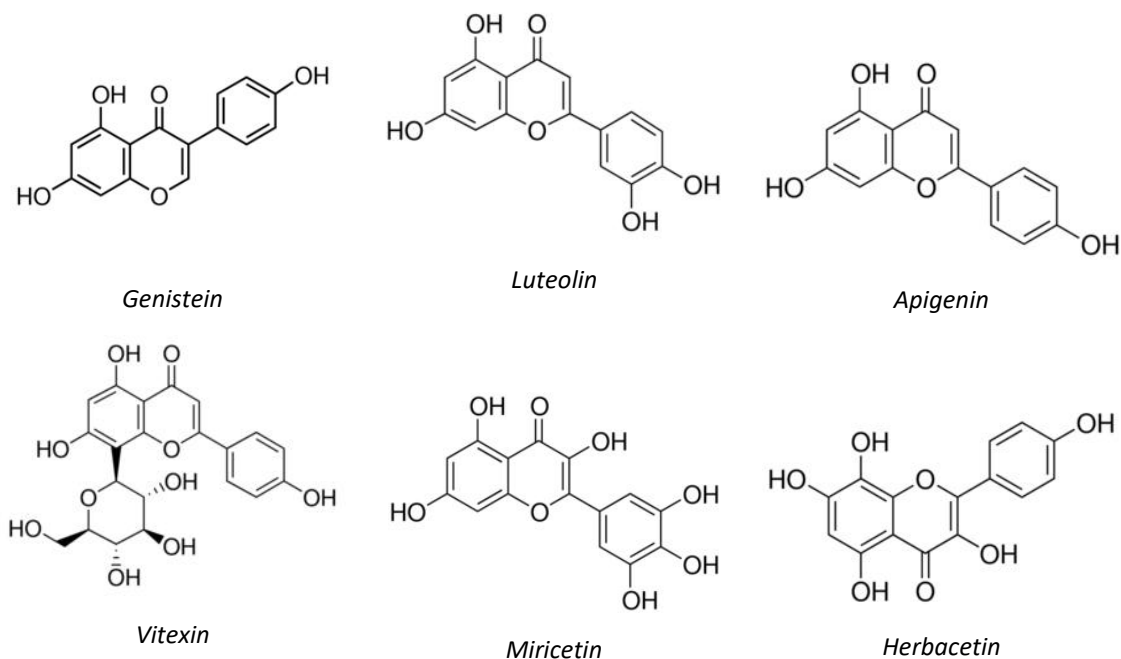
Table 2. Ovarian follicular quantification in different experimental groups.

Parameters	Groups			
	Control 30 dias	Supl. 30 dias	Control 90 dias	Supl. 90 dias
Secondary follicle	6.4±3.7	6.9±2.3	7.6±3.1	6.6±2.4
Antral follicle	6.4±3.1	3.5±2.2*	6.8±2.1	3.2±1.9*
Atresic follicle	6.4±3.1	9.7±3.3	7.2±2.3	14.9±3.8*
Corpus luteum	19.8±4.2	13.5±3.8*	18.7±2.1	8.5±3.7*
Cyst	-	-	-	2.0±0.7*

Values expressed as mean ± standard deviation. Kruskal-Wallis. Dunn's Test. *p<0.05 compared to the control group. Source: Authors.

Figure 4 shows the chemical structure of some flavonoids found in bee pollen.

Figure 4. Chemical structure of some flavonoids found in bee pollen.



Source: Janesar et al. (2017).

4. Discussion

Alves and Santos (2014) evaluated the bee pollen produced in the State of Sergipe in order to determine the main pollen sources visited by *Apis mellifera* L. and to establish an association between the pollen spectrum and climatic variables (temperature and rainfall). The families Asteraceae, Anacardiaceae, Myrtaceae and Rubiaceae had three pollen types each, and Lamiaceae two types. The other families had only one pollen type each. *Cocos nucifera* pollen was present in 100% of the samples. The results found by these authors corroborate those obtained in the present work.

Two samples of dehydrated bee pollen from *Apis mellifera* bee hives were collected in the state of Bahia, and were identified as predominantly from the Fabaceae family. Ash and pH values were found similar to the results shown in the present work. However, the moisture content of their samples was higher than that described in Table 1 (6.50%) and the acidity index was also higher (mean 144.85 mEq/kg) (Caldas et al., 2018).

Six dehydrated bee pollen samples collected in the Vale do Ribeira region, São Paulo, were analyzed, with mean values of 3.08% of ash, 4.97% of lipids, 23.59% of proteins and 2.34% of moisture (Melo et al., 2009). Comparing the results described by the authors with those of the present work, it was found a lower content of ash and proteins, and the content of lipids and moisture was higher, for the pollen of *C. nucifera*. However, all parameters described in Table 1 are in accordance with those established by legislation. For the mineral content, there is no specification in the standard of the Technical Regulation on the Identity and Quality of Bee Pollen (TRIQ) (Brasil, 2001b)

Exposure to xenobiotics during a given day of the cycle can have a marked impact on the endocrine regulation of reproductive function in humans (Ma et al., 2020) and in rats (Kononenko et al. 2020). Bee pollen contains flavonoids in its composition. A subclass of these flavonoids are isoflavones, they have structures and activities similar to human estrogen and are known as phytoestrogens, which can protect the body by blocking estrogen action (Křížová et al., 2019). In bee pollen the isoflavone that appears in higher concentrations is genistein (Ishikawa et al., 2009; Kocote et al., 2018).

The flavonoids present in bee pollen are similar to natural estrogens being able to bind to intracellular estrogen receptors, thus enabling a competitive antagonism, that is, they compete with natural estrogens for receptors in the target organ causing an estrogenic/antiestrogenic effect (Pinto et al., 2010).

Studies by Goldman et al. (2007) report that acute exposure to compounds that interfere with processes in the hypothalamus and are involved in stimulating the secretion of GnRH (the gonadotropin releasing hormone), can block the LH surge (the luteinizing hormone) and alter the cycle. This allows us to suppose that supplementation with bee pollen interferes with the endocrine regulation of the reproductive function through the mechanisms suggested by Pinto et al. (2010). The antiestrogenic effect occurs when certain substances inhibit or modify the action of estrogen, thus inhibiting the effects of normal negative feedback in the hypothalamus and anterior pituitary, which causes an increased secretion of LH, FSH (follicle stimulating hormone) and gonadotropins, as a result ovulation is stimulated (Pinto et al., 2010; Jucá et al., 2020). The estrogenic effect is observed when there is receptor saturation in the target organ, inducing negative feedback in the hypothalamus and anterior pituitary, inhibiting ovulation by decreasing LH, FSH and gonadotropins. These claims are controversial as flavonoids have different structural characteristics and can show various biological activities (Jucá et al., 2020). This may explain the different degree of effect, with some phenolic compounds having an estrogenic effect and others antiestrogenic (Pinto et al., 2010). In the experimental model used in the present work, the histological aspects of ovaries of rats supplemented with monofloral bee pollen of *C. nucifera*, were observed statistically significant decreases in the number of corpora lutea and albicans, increase in atresic follicles in rats supplemented with pollen for 90 days. There was a general increase in the number of follicles in rats in the 30-day challenge group. In contrast, atresic individuals were also included in this increase, and a decrease in ovulation can be observed, as estrogen has a negative feedback action in the anterior pituitary, decreasing the release of gonadotropin during chronic estrogen administration therefore decreases the stimulation of the anterior pituitary to secrete FSH and LH hormones, as occurs in oral contraception. In contrast, endogenous estrogen secretion just before mid-cycle leads to a spike in LH hormone, causing the Graafian follicle to rupture resulting in ovulation (Strauss & Williams, 2019). Studies report that the number of atresic follicles are rare in adult female rats (Asadi et al., 2017), which seems to be a strong indication of the decrease in ovulation due to the effect of pollen supplementation, since the number of atresic follicles found was high.

The low serum concentration of estrogen causes an LH deficiency to occur because with the estrogen peak, there is an LH surge to stimulate ovulation. For this reason it can be explained the presence of ovarian cysts, which can appear by deprivation of LH to some follicles, either through deficiency in LH release or insufficient formation of LH receptors during the follicular maturation phase, thus causing the persistence of one or more cysts. Large cysts secrete progesterone, the extremely high concentration of progesterone in these cysts, can block the positive feedback mechanism of estrogen in LH release (Ebbert & Bostedt, 1993).

The drop in ovarian function demonstrated by the decrease in pre- and post-ovulatory structures suggests that the flavonoids present in bee pollen may have acted as natural estrogens (phytoestrogens) capable of binding to intracellular estrogen receptors, competing with natural estrogens for receptors in the organ. target, leading to negative feedback in the hypothalamus and anterior pituitary, reducing FSH release, responsible for follicular recruitment and LH reduction, and follicular maturation and subsequent ovulation. Kolesarova et al. (2013) showed that the administration of bee pollen in the diet of rats leads to an increase in progesterone in the culture of biopsy fragments from these animals' ovaries. The increase in progesterone can lead to an increase in inhibin and a consequent decrease in FSH and LH levels, which explains the negative effect on ovarian function observed in this work. In addition, bee pollen is a very rich food supplement and there are reports in the literature that ovarian function is extremely sensitive to nutritional status (Kolesarova et al. (2013). Clinical and

experimental studies have shown that declines in ovarian follicular reserves, changes in ovulation rates, and changes in the age of onset of menarche are vulnerable to early life influences (Thakur & Nanda, 2020; Tuoheti et al., 2020).

Sirotkin et al. (2020) made the first observations showing that bee pollen can directly reduce the viability of ovarian cells and positively regulate ovarian cell proliferation, apoptosis and IGF-1 (insulin-like growth factor 1) release. According to the same authors, bee pollen-induced reductions in cell viability may be due to increased apoptosis of ovarian cells, while pollen-induced increase in cell proliferation may be the result of increased IGF-1 release. Furthermore, although all bee pollens evaluated by the authors had similar overall effects on ovarian cell parameters, these observations, according to the authors, are the first to show that the potency and dose-response curves of bee pollen depend on the plant species of origin. The effects of bee pollen on ovarian cells are important information for its use to be considered in nutrition and medicine.

However, to date, the available scientific data are not sufficient to consider this product safe to be considered a medicine.

5. Conclusion

Bee pollen is a widely used food supplement due to the benefits promoted by the bioactive compounds present in it. In addition to the very attractive nutritional power, the presence of phenolics, flavonoids and terpenes is responsible for the antioxidant, antimicrobial, anti-inflammatory activity, possible action on benign diseases and non-cytotoxic prostatic hyperplasia. It was proved that the bee pollen used in this work was from *C. nucifera* and the quality parameters recommended by legislation were in compliance. Regarding the treatment of female rats with pollen, there was a decrease in follicular recruitment, decrease in ovulatory follicles (Graafian) and corpus luteum. There was an increase in atresic follicles and appearance of cysts, showing a negative interference of the bee pollen used on ovarian function. The data show that this interference was time dependent.

The data obtained are very interesting and not very present in the literature. Thus, it is important that new experiments are carried out to prove them.

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