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Research Paper

Androgenic effect of honeybee drone milk in castrated rats: Roles of methyl palmitate and methyl oleate

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ABSTRACT

Ethnopharmacological relevance: Numerous honeybee (*Apis mellifera*) products have been used in traditional medicine to treat infertility and to increase vitality in both men and women. Drone milk (DM) is a relatively little-known honeybee product with a putative sexual hormone effect. The oestrogenic effect of a fraction of DM has recently been reported in rats. However, no information is available on the androgenic effects of DM. The purpose of the present study was to determine the androgen-like effect of DM in male rats and to identify effective compounds.

Materials and methods: A modified Hershberger assay was used to investigate the androgenic effect of crude DM, and the plasma level of testosterone was measured. The prostatic mRNA and protein expression of Spot14-like androgen-inducible protein (SLAP) were also examined with real-time PCR and Western blot techniques. GC–MS and NMR spectroscopic investigations were performed to identify the active components gained by bioactivity-guided fractionation.

Results: The crude DM increased the relative weights of the androgen-dependent organs and the plasma testosterone level in castrated rats and these actions were flutamide-sensitive. DM increased the tissue mRNA and protein level of SLAP, providing further evidence of its androgen-like character. After bioactivity-guided fractionation, two fatty acid esters, methyl palmitate (MP) and methyl oleate (MO), were identified as active compounds. MP alone showed an androgenic effect, whereas MO increased the weight of androgen-sensitive tissues and the plasma testosterone level only in combination.

Conclusion: The experimental data of DM and its active compounds (MO and MP) show androgenic activity confirming the traditional usage of DM. DM or MP or/and MO treatments may project a natural mode for the therapy of male infertility.

1. Introduction

Honeybee products have long been widely used in both folk and clinical medicine. One of the recently most intensively investigated beehive products is royal jelly (RJ), the main food for the queen bee pupa and the queen. The fertility of honeybees is known to be mainly driven by the quality and quantity of their food. The queens consume a high amount of RJ, which contributes to the maintenance of their fertility (Remolina and Hughes, 2008; Kamakura, 2011). These facts and the traditional usage of RJ in the treatment of menopausal symptoms suggested that RJ may possibly have some oestrogen-like effects. In fact, *in vitro* and *in vivo* oestrogenic effects of raw RJ have been described (Mishima et al., 2005) and its isolated effective compounds (*e.g.* 10-hydroxy-*trans*-2-decenoic acid) exhibit weak oestrogen receptor binding affinities (Suzuki et al., 2008).

Drone milk (DM) is a relatively little-known honeybee product that is secreted (similarly to RJ) by the hypopharyngeal and mandibular glands of the worker honeybees. DM, the main component in the drone brood (Mutsaers et al., 2005b), is an essential food of the drone larvae and drone honeybees, and its consumption is presumed to be related to the fertility of drones. Although our knowledge of the effects of DM is very limited, a drone brood preparation is traditionally used in Romania for the rehabilitation and activation of aged people and to treat neuro-vegetative and sexual problems (Bogdanov, 2011). Data have been reported on the hormone-like strengthening effects of the drone

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larvae and brood in Eastern European and Asian folk medicine (Mutsaers et al., 2005a) and Yucel et al. (2011) have described the androgenic and anabolic effects of Apilarnil (drone bee larvae) on male broilers.

The folk medicine experience and the previously described data suggest the putative androgen-like activity of DM. However, this effect has not been proved by any experimental or clinical study carried out in accordance with the principles of evidence-based medicine and research. The aim of our study, therefore, was to investigate the androgen-like effects of crude DM on male rats and to identify the compounds responsible for its androgenic effect.

2. Materials and methods

2.1. Animals

Animal investigations were carried out with the approval of the Hungarian Ethical Committee for Animal Research (permission no.: IV./198/2013) and under the control of the ISO-9001:2008 Quality Management System. The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII. tv. 32.§).

Sexually mature male Sprague–Dawley rats were purchased from the Charles River Laboratories (Budapest, Hungary) and were housed under controlled temperature (20–23 °C), in humidity (40–60%) and light (12 h light/dark regime) regulated rooms. The animals were maintained on tap water and rodent pellet diet (Charles River Laboratories, Budapest, Hungary) *ad libitum*. They were acclimatised to the animal house for 7 days prior to the investigations.

2.2. Investigation of androgenic effect

Androgenic activity was investigated by a simplified Hershberger assay (Freyberger et al., 2007; Tinwell et al., 2007; Moon et al., 2009). Mature male Sprague-Dawley rats (230–240 g) were anaesthetised through the inhalation of 3% isoflurane vapour (Abbott Laboratories, Abbott Park, IL, USA) *via* a Burtons anaesthetic machine (Guardian Industrial Estate, Marden, UK). Castration was performed on animals surgically, and the animals were treated locally with pefloxacine to avoid infective complications. Pefloxacine injection (Peflacine®) was purchased from EGIS Pharmaceutical Industry, Budapest, Hungary.

After 10 days, healthy rats were randomised and assigned to 6 groups ($n=18$/group) by body weight. Body weights were recorded on days 1, 4, 7 and 10 of the study. The animals were treated with the investigated compounds once daily for 10 consecutive days. DM was collected in the early summer by Sándor Polgár (a professional Hungarian beekeeper) and was stored at −20 °C until the beginning of the investigation. DM was diluted with distilled water. Testosterone (Sigma Aldrich, Budapest, Hungary) was dissolved in olive oil. When methyl palmitate (MP) and methyl oleate (MO) (Sigma Aldrich, Budapest, Hungary) were involved in the simplified Hershberger assay, the compound was administered in olive oil by oral gavage. Flutamide (Sigma Aldrich, Budapest, Hungary) was suspended in mucilage methylcellulose (0.5%; Sigma Aldrich, Budapest, Hungary). In a preliminary experiment, DM was administered at three doses (11, 110 or 1100 mg/kg/day), and it was found that the 110 mg/kg/day dose led to increases in the weights of androgen-sensitive organs. The administration protocol for the raw DM administration is outlined in Table 1.

Table 1
Treatments and dosages in castrated rats.

Group	Treatment	Dose	Dosing route
1	Water	1 mL/day	p.o.
2	Drone milk	110 mg/kg/day	p.o.
3	Flutamide	3 mg/kg/day	p.o.
4	Flutamide+drone milk	3+110 mg/kg/day	p.o.
5	Testosterone	0.4 mg/kg/day	s.c.
6	Flutamide+testosterone	3+0.4 mg/kg/day	p.o.+s.c.

p.o.: orally; s.c.: subcutaneously.

One day after the final treatment, the rats were sacrificed under deep isoflurane anaesthesia by exsanguination. Androgen-dependent organs (the glans penis, seminal vesicle, ventral prostate and levator ani muscle) were removed, trimmed free of fat and adjacent tissue and weighed on an HR-202 analytical scale (A&D Instruments, Oxfordshire, UK). Organ weights were expressed as relative weights (organ weight mg/100 g body weight).

2.3. Plasma testosterone assay

Before the exsanguinations, 1 mL of blood was taken from each of 5 animals in each group by cardiac puncture under deep isoflurane anaesthesia. Blood samples were collected in BD Vacutainer tubes (Belliver Industrial Estate, Plymouth, UK) and centrifuged (CR-132, Jouan, Saint-Herblain, France) at 1500*g* for 10 min to separate the plasma. Plasma samples were stored at −70 °C until assay. Plasma testosterone levels were determined with a testosterone EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA). Absorbancies were measured with a SPECTROstar Nano-microplate reader (BMG Labtech, Offenburg, Germany).

2.4. Determination of prostatic Slap mRNA by real-time reverse transcription-PCR

Mature male Sprague-Dawley rats (400–420 g) were castrated as described above. After 7 days, healthy rats were randomised and assigned to 3 groups ($n=5$/group) by body weight. The animals were treated once daily for 5 consecutive days with DM (110 mg/kg by oral gavage) or testosterone (1.67 mg/animal subcutaneously). The control group was treated with the vehicle of the DM (distilled water) by oral gavage. One day after the final treatment, the rats were sacrificed, and ventral prostate tissues were removed and frozen in liquid nitrogen.

The tissues were homogenised mechanically. Total RNAs from tissues were extracted by using a TRIsure Kit (Bioline Reagents Ltd., London, UK). The quality and quantity of the RNA were assessed at wavelengths of 260/280 nm, and all samples exhibited an absorbancy ratio in the range 1.6–2.0.

1 μg of total RNA and the TaqMan RNA-to-C_T 1-Step Kit (Applied Biosystems, Budapest, Hungary) were used for reverse transcription and amplification. The following primers were used: assay ID Rn01756639_g1 (Amplicon Length: 71) for *Slap* (Spot14-like androgen-inducible protein) and Rn99999916_s1 for β-actin as endogenous control (Nishi et al., 2008). RT-PCR was performed with an ABI StepOne Real-Time cycler (Applied Biosystems, Budapest, Hungary). The fluorescence intensities of the probes were plotted against PCR cycle numbers. The amplification cycle displaying the first significant increase in the fluorescence signal was defined as the threshold cycle (C_T).