DOI: 10.2478/v10289-012-0028-4
Vol. 56 No. 2 2012 Journal of Apicultural Science

THE EFFECT OF AMPHOTERICIN B ON THE LIFESPAN, BODY-SURFACE PROTEIN CONCENTRATIONS, AND DNA METHYLATION LEVELS OF HONEY BEES (*Apis mellifera*)

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Received 14 September 2012; accepted 19 November 2012

S u m m a r y

Three groups of caged bees were fed with sugar syrup (the control), sugar syrup supplemented with amphotericin B (AmB) in a dose of 0.5 mg/ml, and sugar syrup with AmB in a dose of 0.25 mg/ml. Amphotericin B shortened the life span of the bees and reduced the level of global DNA methylation compared to the control, however, it increased the body-surface protein concentrations. In the hindguts of the bees, there were found AmB deposits. Honeybees appear to be a useful model for studying the side effects of anti-fungal AmB therapy. Among other things, epigenetic changes and senescence acceleration are considered to be the side effects of the therapy.

Keywords: amphotericin B, *Apis mellifera,* DNA methylation, life-span, protein concentration.

INTRODUCTION

Amphotericin B (AmB) is a macrolide polyene antibiotic which is very efficient in the treatment of deep-seated mycotic infections but has toxic side effects. Nevertheless, the application of AmB to treat several mycoses of internal organs occurring in the aftermath of AIDS (Konopka et al., 1999; Krukowski et al., 2010) and for internal organ transplants is experiencing its renaissance (Hartsel and Bolard, 1996). We simply have to choose the "lesser evil". It is accepted, that the biological effect of AmB is based on the interaction with cellular membranes and particularly on the formation of transmembrane pores that are able to seriously affect the physiological ion transport. However, precise mechanisms responsible for both therapeutic and toxic side effects of AmB are still not fully understood. Numerous side effects, such as nephrotoxic activity, hepatotoxic activity, and in higher doses - neurotoxic and hemolytic activities are induced by AmB (Gallis et al., 1990). One of the main

problems of AmB treatment is related to its aggregation (Espada et al., 2008; Gagoś et al., 2011). It was recently found by Gagoś et al. (2011), that the formation of AmB-Cu^{2+} complexes is linked to the disaggregation of AmB. This may facilitate practical applications of AmB.

There are two strategies in fighting apian mycosis, i.e. the use of medicine and taking advantage of apian behavioural resistance (Chorbiński, 2004). The chemical agents in use include disinfectants, organic acids, plant extracts, and antifungal drugs - e.g. AmB (Kostecka and Niewiadomy, 2010). However, veterinarian regulations do not allow the use of antibiotics in *Nosema* spp. treatment in the EU. On the other hand, there is an increasing problem with the *Nosema ceranae* invasion in Europe (Ptaszyńska et al., 2012a, b). There are kept bee populations that must not be lost (e.g. De la Rua et al., 2009). In order to better use AmB in humans, and also to enable the restricted use of AmB in bees (the lesser evil), we have to learn more about the functioning of this drug in living organisms.

Apis mellifera has been found to be a unique model for some biochemical and genetic studies of vertebrates and humans (Burzyński, 2009; Paleolog et al., 2011; Strachecka et al., 2012c). Contrary to the commonly used *Drosophila*, it has the advantage of a large organismal size (Munch et al., 2008). It is not only the genome content that determines the functions of living organisms. An important role is also played by the gene-silencing molecular switches - the epigenome that is sometimes more important for heredity than the DNA code (Burzyński, 2009). Lyko and Maleszka (2011) have shown that honeybees are a good model for the epigenetic study of vertebrates. They can store epigenetic information and perform DNA methylation processes (basic for epigenetic genome regulation) in a way similar to humans (Kucharski et al., 2008; Maleszka, 2008).

Considering the global problems with mycosis, and considering the only partially known harmful side effects of the AmB therapy in humans, and especially the lack of information about the consequences of AmB use for genome methylation (epigenetic regulation), we studied how amphotericin B affects the life span, global DNA 5-methylocytosine (m5C) content, and cuticle surface protein concentration in bees. We also focused our study on the location of amphotericin B molecules in the body of honeybees, using honeybees as model organisms. The studies also offered the opportunity to increase the chances of a restricted use of AmB in honeybees.

MATERIAL AND METHODS

Three bee groups were set up: 10 wooden cages (12 x 12 x 4 cm) with 50 one-day-old worker bees in each of the groups. The cages had glass front screens but they had no combs or wax foundations inside. The sugar syrup (1:1) was administered *ad libitum* via inner feeders. In the first group (AmB-25), the syrup was supplemented with amphotericin B (AmB; Sigma Aldrich) at a concentration of 0.25 mg/ml, in the second group (AmB-50) at a concentration of 0.50 mg/ml, whereas in the third group which was the control, AmB was not added. The cages were kept within an air-conditioned chamber (26°C and 65% RH). Every two days, the feeders were replenished with the syrup. Dead workers were removed from the cages and divided into 4 sets: 1 - to assess *Nosema* spp. infestation, 2 - to detect AmB aggregations in the apian bodies, 3 - to isolate DNA and analyse its methylation, 4 - to determine protein concentration. Set 1 and set 2 were directly processed. The other two sets were frozen (-25°C). Each set contained approximately 125 bees.

Set 1: The extent of *Nosema* spp. infestation was assessed as described by Pohorecka (2004).

Set 2: The workers were X-rayed using the Faxitron MX-20 system for biological material structures. When irradiated, AmB aggregations should emit a fluorescent glow. Subsequently, the workers treated with AmB-50 and AmB-25 were incised

under the Olympus SZX16 Stereo Zoom Microscope: (camera: Olympus DP72). We looked for aggregations of AmB in the apian bodies, and when they were found, we photographed them.

Set: 3: After defrosting, DNA was extracted from the workers' heads and thoraces using the DNeasy Blood & Tissue Kit (Qiagen) according to the producer's procedure. Extracted DNA samples were stored at -25°C and subsequently modified by treatment with Capture Antibody using the MDQ1-96RXN Imprint® Methylated DNA Quantification Kit (Sigma; producer's procedure).

Set 4: The workers were defrosted. Next, they were rinsed in 10 ml of distilled water for 20 seconds to remove impurities. The Lowry method modified by Schacterle and Pollack (1973) was used. Since the rinsings were found to contain no proteins, they were discarded. Subsequently, the workers were put into test tubes (5 insects in each; 24 samples per group), 10 ml of a 1% detergent: water solution (Triton X-100) was added and the bees were shaken/rinsed again for 10 min. at 3400 rpm. After filtrating each of the samples through Miracloth, concentrations of the proteins isolated from the apian cuticles were determined as described above.

The significance of the AmB effects and differences between the average trait values were investigated with one-way ANOVA and LSD procedures using SAS statistical software (SAS Institute Version 9.13., 2002-2003 license 86636). The statistical calculations were performed using numbers transformed according to arcsine transformation.

RESULTS

Compared to the control group, the bees treated with AmB-25 and AmB-50 lived shorter (Tab. 1) but they were not infested with *Nosema* spp. (AmB-50: 0 ± 0 [120]; Amb-25: 0 ± 0 [125]; the control: 29 ± 2.01 [120] spores).

The X- ray system detected AmB as bright, shiny spots in the AmB-25 and AMB-50 workers (Fig. 1A). It was also found as yellow spots/clusters in their hindguts (Fig. 1B) with the Stereo Zoom Microscope. There was no absorption (no AmB aggregations) in the cavities of the body, haemolymph, etc.

The level of global DNA methylation in the one-day-old workers (the first day of the study) was the same in all the groups (AmB-50: 6.25 ± 0.002 [10]; AmB-25: 6.25 ± 0.003 [10]; the control: 6.25 ± 0.003 [10]). In comparison with the control, AmB significantly decreased DNA

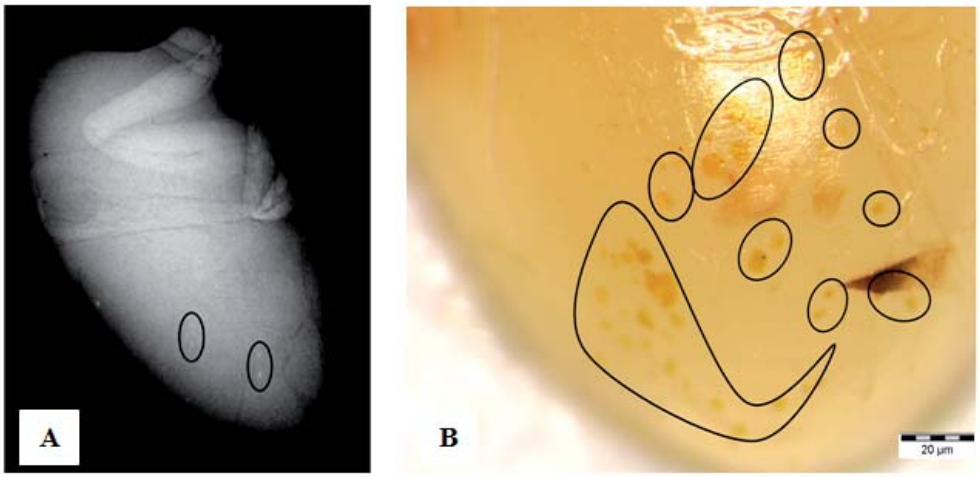

Fig. 1. Location of amphotericin B (AmB) in the hindgut of the *A. mellifera* workers.
A: X-ray system image; B: Stereo zoom microscope image.

Journal of Apicultural Science

Table 1.

Mortality of caged worker bees expressed
in percentiles of the life-span distribution

Group	n [cages]	The day on which the following percentages of the caged workers were still alive:							
		70%		50%		30%		0%	
		$\bar{x}$	± se	$\bar{x}$	± se	$\bar{x}$	± se	$\bar{x}$	± se
The control	10	16b	1.03	21b	0.80	25b	0.79	36b	0.76
AmB-25	10	13a	0.54	16a	0.70	20ab	1.03	28a	1.26
AmB-50	10	12a	0.87	15a	0.90	19a	1.12	27a	1.96

Various lowercase letters - the differences are statistically significant at $P \leq 0.05$ for comparisons within the columns (between the control and AmB-25 and AmB-50);
$\bar{x}$- mean value;
±se - standard error;
AmB-25 - the workers were treated with 0.25 mg/ml amphotericin B;
AmB-50 - the workers were treated with 0.50 mg/ml amphotericin B.

Table 2.

Global DNA methylation level (%)
in workers treated with amphotericin B (AmB)

Group	n [workers in each period]	1-week-old workers		2-week-old workers		4-week-old workers		5-week-old workers	
		$\bar{x}$	± se	$\bar{x}$	± se	$\bar{x}$	± se	$\bar{x}$	± se
The control	30	6.98a	0.004	7.46a	0.005	14.96a	0.004	22.49a	0.005
AmB-25	30	6.12b	0.002	6.76b	0.005	10.7b	0.003	10.8b	0.005
AmB-50	30	5.91c	0.004	1.15c	0.005	3.72c	0.004	4.41c	0.004

Various lowercase letters - the differences are statistically significant for comparisons in the columns (between the control and AmB-25 and AmB-50) at $P \leq 0.05$;
$\bar{x}$- mean value;
±se - standard error;
AmB-25 - the workers were treated with 0.25 mg/ml amphotericin B;
AmB-50 - the workers were treated with 0.50 mg/ml amphotericin B.
No statistically significant differences were observed between the samples in a specific period (2-3 samplings per week).

Table 3.

Protein concentrations (mg/ml) on the body surface
of *A. mellifera* workers treated with amphotericin B (AmB)

Group	n [workers in each period]	1-day-old - 2 -week-old workers		3-4 - week-old workers		5 - week-old workers	
		$\bar{x}$	± se	$\bar{x}$	± se	$\bar{x}$	± se
The control	40	0.104b	0.03	0.122b	0.04	0.026b	0.02
AmB-25	40	0.225a	0.05	0.289a	0.07	0.096a	0.02
AmB-50	40	0.124ab	0.06	0.189ab	0.05	0.048ab	0.02

Various lowercase letters - the differences are statistically significant for comparisons in the columns (between the control and AmB-25 and AmB-50) at $P \leq 0.05$;
$\bar{x}$ - mean value;
± se - standard error;
shaded - the highest values of protein concentration;
AmB-25 - the workers were treated with 0.25 mg/ml amphotericin B;
AmB-50 - the workers were treated with 0.50 mg/ml amphotericin B.
No statistically significant differences were observed between the samples in a specific period (7-8 samplings).

methylation levels (Tab. 2).The influence of AmB on methylation increased along with the duration of the treatment and AmB doses.

Increased protein concentrations were observed in all the groups between the 3rd and 4th week (Tab. 3). The concentrations were higher in the case of AmB-50 and AmB-25 in comparison with the control, regardless of the AmB doses.

DISCUSSION

Amphotericin B is a life-saving antibiotic in human mycosis and, as confirmed by our study, an anti-*Nosema* spp protector in honeybees. Unfortunately, AmB is toxic to humans and forms concrements in the liver, kidneys, and the brain (Gagoś et al., 2011). As we found, AmB is also deposited in honeybee hindguts (Fig. 1). Therefore, honeybees seems to be a good model for studying the mechanisms of this process. The new finding of this study was that AmB affects epigenetic control in the apian genome by reducing the global DNA methylation level (Tab. 2), probably by disturbing the methylation/demethylation equilibrium. A similar interrelationship was observed by Bardini et al. (2003) in a model plant *Arabidopsis thaliana,* following kanamycin administration. Changes in the genome methylation influence protein inhibitor synthesis and methylotransferase activity. This results in an altered metabolism (Bardini et al., 2003), with an effect on the lifespan (Burzyński, 2009). Senescence is a complex process connected with changes in many elements of numerous key metabolic pathways, and the honeybee is a unique known model in gerontology (Munch et al., 2008 and references therein). We found that AmB not only changed the DNA methylation level but also the lifespan of honeybees (Tab. 1). Hence, senescence acceleration may be considered as a side effect of treatment with AmB and probably also a side effect of treatment with other antibiotics. One of senescence symptoms is a reduction of protein synthesis, which was observed in this study in 5-week-old workers (Tab. 3). We observed effects of AmB treatment on the apian cuticle protein concentrations. A dosage effect was additionally identified. A higher effect was observed for smaller doses in comparison with larger ones. This effect was also observed by Kang et al. (2002) in *Drosophila*. It turns out, that activity of the cuticle protein system may be alerted as a consequence of antibiotic therapy, which is similar to the results obtained during acaricide therapy against *Varroa destructor* in honeybees (Howis et al., 2012; Strachecka et al., 2012a, b).

CONCLUSION

The honeybee proved a useful model for studies of the side effects of AmB therapy. The side effects of the therapy include epigenetic changes and senescence acceleration, accompanied by changes in the protein concentration.

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WPŁYW AMFOTERYCYNY B NA DŁUGOŚĆ ŻYCIA, STĘŻENIE BIAŁEK NA POWIERZCHNI CIAŁA I POZIOM METYLACJI DNA U PSZCZÓŁ (*Apis mellifera*)

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S t r e s z c z e n i e

Trzem grupom pszczół w klatkach podawano syrop cukrowy (grupa kontrolna), syrop cukrowy z dodatkiem amfoterycyny B (AmB) w dawce 0,5 mg/ml oraz syrop cukrowy z AmB w dawce 0,25 mg/ml. AmB wpływała na skrócenie długości życia pszczół oraz obniżała poziom globalnej metylacji DNA w porównaniu do grupy kontrolnej, jednocześnie zwiększała stężenie białek na powierzchni ciała. AmB odkładała się w jelicie tylnym pszczół. Pszczoła miodna okazała się użytecznym modelem dla badań efektów ubocznych terapii antygrzybiczej z zastosowaniem AmB. Między innymi, za efekty uboczne takiej terapii uważa się zmiany epigenetyczne i przyspieszenie procesów starzenia.

Słowa kluczowe: amfoterycyna B, metylacja DNA, stężenie białka, długość życia, *Apis mellifera.*