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Research Article

Effect of Acyclovir on the Microbial Contamination in the Artifical and Natural Diets for Rearing of *Galleria mellonella* L. larvae

Asiklovirin Galleria mellonella L. Larvalarının Yetiştirilmesinde Kullanılan Yapay ve Doğal Besinlerdeki Mikrobiyal Kontaminasyona Etkisi

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Abstract

The effects of clinically important antiviral agent, acyclovir, on microbial contamination sources in the artificial (Bronskill diet) and natural diets (dark old honeycomb) used for rearing of a serious pest in the honey bee hives, greater wax moth *Galleria mellonella* L. The larvae were reared on diets containing different concentrations of acyclovir (0.001-3.0 %). Control larvae were reared on diet without acyclovir. The diet with 1.0% of acyclovir had no significant negative effects on biological parameters of immature stages and adult stage. Therefore, dietary antimicrobial effects of this safe concentration on insect were tested. Antimicrobial effects varied with the rearing media, initial microbial sources in diets and microorganism types. Acyclovir at 1.0 g in artificial diet was significantly capable of reducing the number of yeast and molds from 6000 ± 565.6 and 800 ± 20.6 CFU to 800 ± 35.3 and 50 ± 2.3 CFU respectively. Proliferation of *Stahylococcus aureus* (3200 ± 141.4 CFU) inhabiting this diet were completely inhibited by 1.0 g of acyclovir. Coliform bacteria are not seen in the artificial diet. This concentration of acyclovir completely inhibited proliferation of coliform in natural diet while the artificial diet lacks coliform bacteria. Acyclovir at a level of 1.0% was also of capable of preventing the bacterium, mold and yeast growth in both artificial diet and natural food. We infer from these results that the importance of determining the antimicrobial effect of an antimicrobial agent before adding it as a food additive to the diet during laboratory rearing of insects.

Keywords: Acyclovir, Antimicrobial effect, Artificial and natural diets, *Galleria mellonella*

Öz

Klinik öneme sahip antiviral asiklovirin bal arısı kovanlarının önemli bir zararlısı olan büyük bal mumu güvesi *Galleria mellonella* L.'nın larvalarını laboratuvar şartlarında yetiştirmek için kullanılan yapay (Bronskill besini) ve doğal besinlerdeki (kabartılmış boş koyu renkli eski petek; kuluçka peteği) mikrobiyal kontaminasyon kaynaklarına etkisi incelendi. Larvalar asiklovirin farklı konsantrasyonlarını (% 0.001-3.0) içeren besinler ile yetiştirildi. Kontrol grubunda larvalar asiklovir içermeyen besin ile yetiştirildi. Asiklovirin % 1.0'lık konsantrasyonunu içeren besinin böceğin ergin öncesi ve ergin evredeki biyolojik parametreleri üzerinde önemli bir olumsuz etkiye sahip olmadığı belirlendi. Bu sebepten dolayı böceğe karşı olumsuz etkiye sahip olmayan bu konsantrasyonun besinlerdeki antimikrobiyal etkisi incelendi. Antimikrobiyal etki beslenme ortamına, mikrobial kaynakların başlangıçtaki yoğunluklarına ve mikrobiyal organizmaların türüne göre değişti. Asiklovirin % 1.0'lık konsantrasyonu yapay besindeki maya ve küf sayısını sırasıyla 6000 ± 565.6 ve 800 ± 20.6 CFU'den 800 ± 35.3 and 50 ± 2.3 CFU'e önemli derecede düşürürken, *Stahylococcus aureus* (3200 ± 141.4 CFU) bakterisinin çoğalmasını tamamen önledi. Yapay besinde koliform bakteriler bulunmazken doğal besinde 1100 ± 84.8 CFU olarak tespit edilen koliform bakterilerin çoğalması tamamen önlendi. Asiklovirin % 1.0'lık konsantrasyonunun besin ortamlarındaki bakteri, küf ve maya kontaminasyonunu önlemede etkili olduğu belirlendi. Bu çalışma bir antimikrobiyal maddenin böceklerin laboratuvar şartlarında yetiştirilmesi sırasında katkı maddesi olarak besine ilave edilmeden önce antimikrobiyal etkisinin belirlenmesi gerektiğinin önemini belirtmektedir.

Anahtar Kelimeler: Asiklovir, Antimikrobiyal etki, Yapay ve doğal besinler, *Galleria mellonella*

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1. Introduction

Mass rearing of insects in the laboratory conditions for any purposes has introduced a number of problems of microbiological origin such as disease transmission, sterilization techniques for eggs, and control of contaminating microbial organisms. Microbial control in insects diets and cultures is one of the most troublesome components of mass insect rearing. The conditions of temperature, humidity and nutritional situation of artificial diets for *Galleria mellonella* are ideal for microbial growth. Yeast and molds are the most commonly encountered contaminants and of the most difficult to control (Gifawesen et al. 1975, Bucher and Williams 1967, Jarosz 1981). Availability and quality of larval food affect the physiological traits in immature and adult stages of pyralid moths (Bell 1975, Ryne et al. 2004). Therefore, antibacterial and antifungal agents which are either identical to or related to those administrated to humans are used in artificial diet of lepidopteran (Deecher et al. 1989, Jarosz 1981, Jarosz 1989, Lee et al. 1999) and other insects (Bass and Barnes 1969, Singh and House 1970, Büyükgüzel and Yazgan 2002, Büyükgüzel 2001a, b, Alverson and Cohen 2002, Inglis and Cohen 2004) for subtherapeutic purposes with the goal of growth promotion, enhanced feed efficiency, microbial control, obtained aseptic larvae for physiological investigation. These studies demonstrated that antimicrobial agents can have also detrimental effects on development and health of insects.

G. mellonella L. is a pest, resulting important economic damage to honey-bee combs during storage and also to weak honey-bee colony in hives. The wax moth larvae provide an important tool for investigation the pathogenesis of a wide range of microbial infections including mammalian fungal and bacterial pathogens (Chadwick et al. 1990, Jander et al. 2000, Ramarao et al. 2012) aside their usage for determining the insecticidal effects of chemicals for altenative pest insect management purposes. The rearing of *G. mellonella* in laboratory condition is easy and relatively cheap. Larvae are relatively large, thus enabling application defined doses of bacteria by injection or *per os* experiments. Larvae can be reared at high temperatures (37 °C) allowing experiments that mimic a mammalian host (Réjasse et a l. 2012).

Formalin and sodium hypochlorite as traditional food antiviral additive was commonly used for control viral contamination in the artificial rearing diets (Vail et al. 1968). These antivirals were found to have significant negative effects on some biological parameters of some economically important insects (Alverson and Cohen 2002). Sun (1981) indicated that formalin is carcinogen and poses other health risks for employees who are exposed to it during preparation and handling of the diet. Acyclovir is an acyclic guanine nucleoside analogue that is videly used clinically as a new brand antiviral antiherpetic agent and show low toxicity in vertebrates (De Clerco and Holy 1979) This antiviral agent is widely distributed to tissues and body fluids and is selectively phosphorilated in virus-infected cells to a monophosphate ester by viral thymidine kinase. Followed by further phosphorilation to a triphosphate ester which is inhibits virus DNA polymerase and competes with the natural deoxyguanosine triphosphate, for incorporation into viral DNA (Elion 1993). Efforts focused on finding an alternative antiviral agent and determining their safe levels that adequately controls viral and other microbial contamination while not significantly affecting the life table parameters of the insects. This study demonstrated that antimicrobial activity of safe level of acyclovir which has any detrimental effect on biology of *G. mellonella* larvae reared on artificial and natural diets.

2. Material and Methods

2.1. Insect Stock Culture

Larvae and pupae of greater wax moth *G. mellonella* (Lepidoptera: Pyralidae) were collected from infected hives in apicultural area of Zonguldak, Turkey and newly emerged adults were used to maintain the stock culture on artificial diet, at 30 ± 1^{0}C, 65 ± 5% humidity in constant darkness. The synthetic diet described by Bronskill (1961) was used for rearing the larvae of the insect in maintaining the stock culture. Diet preparation, placing the larvae onto diet and rearing techniques are given in our previous studies (Büyükgüzel et al. 2013, Çalık et al. 2015).

2.2. Feeding Experiments

Newly emerged two or three females were placed into plastic cups (ORLAB, L190030, 35x55 mm, Ankara, Turkey) with screen lid for oviposition as described by Al-izzi et al. (1987). These cups were kept in the same laboratory conditions as mentioned for the stock culture. Females were allowed to oviposit in cups. All egg for experimental uses was collected within 24 h. Newly hatched larvae were transferred by means of fine brush to atificial diets containing different levels of acycvlovir, and natural diets treated with this antiviral agent for feeding experiments.

Effects of acyclovir on microbial contaminants in two larval diets of greater wax moth, *G. mellonella* were determined.

First one is artificial diet of Bronskill (1961) commonly used to rear *G. mellonella* in the laboratory conditions. The diet contained 420 g of bran, 150 mL of filtered honey, 150 mL of glycerol (Merck, Darmstadt, Germany), 20 g of ground old dark honeycomb and 30 mL of distilled water. Natural diet is empty old dark honeycombs (Brood Combs). They are obtained from apiary in Zonguldak, Turkey

Acyclovir, acycloguanosine, (100.76%) was gift from Biofarma Drug Ltd. (Samandıra, Istanbul, Turkey). Acyclovir was tested because it was the first clinically important antiviral agent used in artificial rearing of this insect except for traditional antiviral agents formaline and sodium hypochloride (Vail et al. 1968). The tested levels of acyclovir were directly incorporated into diets as g of 100 g diet and thoroughly mixed with other components of the diets for feeding experiments. In treating natural diet, different concentrations of this antiviral agent were prepared by dissolving in distilled water as g/100ml and sprayed into old dark combs.

One-day-old first instar larvae were obtained from stock culture as mentioned above. The larvae were placed in rearing jars containing either 0.001, 0.01, 0.1, 1.0 and 3.0 g of diet. Control larvae were reared on diet without acyclovir. For natural diets, desired concentrations of acyclovir were prapared by distilled water as g/100 ml. The concentrations were 0.01, 0.1, 1.0 and 3.0 g of 100 ml distilled water. A 5 ml of an appropriate concentration of acyclovir per approximately 20 cm^2 of combs surface was applied by means of spray bottle. To maintain the normal conditions of the wax moth development, the honeycomb was covered with another honeycomb, also uniformly sprayed over both surfaces with acyclovir solutions. The honeycombs were allowed to dry before inoculations of first instar larvae. Control larvae were fed on old dark honeycombs sprayed with an equal volume of distilled water. The methods in feeding with natural diet treated were modified from that of Jarosz (1981). Rearing experiments with artificial and natural diets to determine the safe level of acyclovir on the insect were conducted under the same laboratory conditions as in the case of stock culture. Each experiment was replicated four times with ten larvae per replication.

2.3. Antimicrobial Effects

Acyclovir was tested to determine their ability to supress or control the growth of microorganisms in the diet. Our preliminary experiments in this study demonstrated that acyclovir at 1.0 g in artificial and natural diet was safe level on the survival, development (unpublished data). Furthermore our current data showed that this acyclovir concentration also is safe level on adult longevity and egg production of the insect compared to other tested levels. To determine effects of 1.0 g of acyclovir on microbial flora of artifical and natural diets, microbiological test for bacteria, yeast and mold which are abundantly present in artificial diet was performed on the diets (Chapman et al. 1937, Mallmann and Darby 1941, Galloway and Borges 1952). Because other levels of acyclovir tested caused a decrease in survival of the insect, microbiological test of the diets with these levels was not performed. After the active feeding period of the larvae, the cups containing remainder artificial and natural diets were tightly covered. The cups was cleaned with ethanol (95%) for preventing surface contamination. Under aseptic condition, 1 g samples of medium was mixed in 9 ml of sterile distilled water and immediately plating a sample of suspension on the microbiological diets. *S. aureus* which is common symbiotic bacterium in insect gut, coliform bacteria which are indicator of external contamination of dietary components especially stored honeycombs, and yeast and moulds which are serious microbial contaminants in artificial diets of insects were taken as the most important criteria for evaluating antimicrobial effects of acyclovir. Total number of selected microbial organisms was expressed as colony forming units (CFU) per g of diets. Microbiological tests were replicated two times.

2.4. Statistical Analysis

Data on the antimicrobial effects of acyclovir in the diets were compared with *t*-test (Sokal and Rohlf 1969). When *t* exceed their 0.05 value, the differences were considered significant.

3. Results

Effects of acyclovir on microbial organisms of artificial and natural diets were shown in Table 1-2. Total count of yeast and molds was average 6000 ± 565.6 and 800 ± 23.6 CFU respectively per g of artificial diet used as control diet in the current feeding experiments. Acyclovir at 1.0 g in this diet was significantly capable of reducing the number of yeast and moulds to 800 ± 35.3 and 50 ± 2.3 CFU respectively. Proliferation of *Stahylococcus aureus* (3200 ± 141.4 CFU) inhabiting this diet were completely inhibited by 1.0 g of acyclovir. Coliform bacteria are not seen in the artificial diet (Table 1).

A large number of yeast and molds were recorded in the natural diet, old dark honeycombs. Total counts of these microbial organisms were reduced to only 500 ± 106.1 for yeast and 4000 ± 212.1 CFU for molds when 1.0 g of acyclovir was applied into diet. The natural diet also contained high number of *S. aureus* (7500 ± 353.5 CFU) compared to artificial diet. Contrary to artificial diet, coliform bacteria were seen at a number of 1100 ± 84.8 CFU in natural diet, honeycombs. Acyclovir at 1.0 g suppressed growth of all coliform bacteria whereas this level reduced the number of *S. aureus* from 7500 ± 353.5 to 1500.0 ± 141.4 CFU (Table 2). Although this reduction in the number of *S. aureus* are statistically important, the cabability of acyclovir at 1.0 g in suppressing *S. aureus* contamination in natural diet is unsufficient (Table 2).

4. Discussion

Our study showed that the concentration of acyclovir which is safe for adult biological fitness of *G. mellonella* is capable of preventing the bacterium, mold and yeast growth in both artificial and natural diets. We infer from our results that use of acyclovir and other possible nucleoside analogs appears to be compatible in artificial rearing of insects with traditional antiviral agents such as formalin and sodium hypochloride for pest management programs or any other purposes (Vail et al. 1968).

The antimicrobial agents in the diets must not only be effective in microbial inhibition, they must also be safe to the target insects reared on artificial diets as suggested for Singh and House (1970). The conditions of temperature and humidity under which insects are raised are ideal for microbial growth including mold, fungus, yeast and bacteria. *Aspergillus niger* is one of the most commonly encountered contaminants whose control is the most difficult (Singh and Bucher 1971, Gifawesen et al. 1975). *G. mellonella* culture at our laboratory experiences periodic contamination problems with fungi and bacteria in the larval rearing diet (personel observations). Researchers showed that microorganisms can have detrimental effects on development and survival of insects (Sikorowski and Thompson 1984, Singh and House 1970). At concentrations as safe levels determined by Singh and House (1970), many antimicrobials had no condiderable effects on development and survival in larval and pupal stages of *Agria affinis* (Fallén). Bass and Barnes (1969) found that 100% of white-fringed beetle, *Graphognathus* spp larvae died within 10 d when a number of different antifungal agents were used in concentrations high enough for fungus control. This study reported that some antimicrobial agents found to be relatively non-toxic to 1st-instar larvae at low concentrations, however, these agents or combinations of these agents at non-toxic concentrations

Table 1. Effects of acyclovir on microbial organisms in artificial diets for rearing *G. mellonella* larvae.

Source of microbial contamination in the diets	Artificial diet*	
	Control	Acyclovir
Staphylococcus aureus	3200.0 ± 141.4	-
Coliform bacteria	-	-
Mold	800.0 ± 23.6 (x)	50.0 ± 2.3 (y)
Yeast	6000.0 ± 565.6 (x)	800.0 ± 35.3 (y)

*Average of two replicates. Means (±S.D) followed by the different letter [(x) (y)] in the same line are significantly different from each other, $P < 0.05$ (t-test). Control diets (without acyclovir), Amount of the microorganisms in the diets was given as Colony Forming Unit (CFU/g of diet).

Table 2. Effects of acyclovir on microbial organisms in natural diets for rearing *G. mellonella*.

Source of microbial contamination in the diets	Natural diet*	
	Control	Acyclovir
Staphylococcus aureus	7500.0 ± 353.5 (x)	1500.0 ± 141.4 (y)
Coliform bacteria	1100.0 ± 84.8	-
Mold	∞	4000.0 ± 212.1
Yeast	∞	500.0 ± 106.1

*Average of two replicates. Means (±S.D) followed by the different letter [(x) (y)] in the same line are significantly different from each other, $P < 0.05$ (t-test)· Control diets (without acyclovir), Amount of the microorganisms in the diets was given as Colony Forming Unit (CFU/g of diet), ∞Very high number.

levels failed to prevent microbial growth. These studies and others emphasize the importance of determining the toxicity of an anitmicrobial agent to a particular insect species before adding it to the diet. The additive tested in current study was the first clinically used acyclovir and currently widely used in treatment of human and animal viral desease. On the other hand, antimicrobial agents intended to preserve insect diets and insect culture well being must also obey rules for human safety principal suggested by Alverson and Cohen (2002).

Coliform bacterial populations as a level of 1100 ± 84.8 CFU in natural diet without acyclovir are present. Bacterial populations including *S. aureus* were also high in natural diets without acyclovir (7500 ± 353.5 CFU) but they were significantly higher by about 5-fold than in diets containing antiviral agents (1500 ± 141.4 CFU). In the artificial diet, the acyclovir concentration (1.0%) was completely effective to supress bacterial contamination (Coliforms and *S. aureus*) but it also has decreasing effects on mold and yeast in the artificial and natural diets. Similar to our results, significant reductions in numbers of total aerobic faecal microorganisms were noted in American cockroaches fed antibiotics daily (Klowden and Greenberg 1977). Moreover, various antibiotics in diets of boll weevil, *Anthonomus grandis* Boheman, reduced the number of bacteria and fungi however, they had no significant negative effects on egg production and hatchability (Sikorowski et al. 1980). In the ligh of the results of the previous studies, although dietary concentration which is safe on insects would be low, possible effects of this antiviral compound on the target insect and other nontarget organisms must be considered. The importance in use of artificial diets with antimicrobials is the production of high-quality insects by ameliorating the nutritional quality of diets. Therefore, additional studies are required to elucidate the effect of acyclovir on *G. mellonella* adults fitness in the term of longevity, fecundity and fertility and this may become an important issue with regard to the development of novel drugs with selective insecticidal potential.

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