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Physiological and Biochemical Effects of *Klebsiella oxytoca* Infection on Model Organism *Galleria mellonella* L.

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ABSTRACT

In this study, the physiological and biochemical response of model insect *Galleria mellonella* due to *Klebsiella oxytoca* infection was investigated. Changes in metabolic enzyme activities such as aspartate transferase (AST), alanine transferase (ALT), alkaline phosphatase (ALP), amylase (AMYL) and gamma glutamyl transferase (GGT) occurred due to *K. oxytoca* infection. In addition, amounts of non-enzymatic antioxidant such as uric acid (UA), bilirubin (BIL) and albumin (ALB) were altered in *G. mellonella* hemolymph due to infection. The results obtained from this study showed that *G. mellonella* can be used as a model organism in the evaluation of the physiological effects of *K. oxytoca* infection on the host.

Key words: Galleria mellonella, model insect, antioxidant responses, infection.

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INTRODUCTION

Klebsiella species are nosocomial pathogens with high virulence. Although they usually infect immunocompromised individuals, these isolates make treatment difficult because they are resistant to many antibiotics (Bassetti et al, 2018; Rønning et al, 2019). *Klebsiella oxytoca* is a gram-negative bacteria associated with nosocomial infections. In recent years, the high resistance of *K. oxytoca* against antibiotics used especially in urinary tract infections has increased the interest of researchers in this pathogen (Tominaga 2018; Hu, Wei, Feng, Xie, & Zong, 2019).

In recent years, invertebrate experimental animals have been used to study bacterial infection and host interactions (Shaik, Mishra, Sehadová, & Kodrík, 2020; Lapointe, McCarthy, Dunphy, & Mandato, 2020). In addition, invertebrate infection models are frequently used in the fields of medicine, pharmacy and veterinary medicine (Matsumoto, Ishii, Shimizu, Kawamoto, & Sekimizu, 2017; Ishii, Matsumoto, Yamada, Abe, & Sekimizu, 2017; Singulani, Scorzoni, De Oliveira, Marcos, & Assato, 2018). In particular, *Galleria mellonella* (Lepidoptera: Pyralidae) larvae have been used as model organisms in many studies on bacterial pathogenicity or the efficacy of antibiotic treatments (Rossoni et al, 2017; Shaik et al, 2020; Lapointe, McCarthy, Dunphy, & Mandato, 2020). The large surface area of *G. mellonella* larvae and the easy isolation of hemolymph provide advantages for physiological and biochemical studies (Andrejko, Zdybicka-Barabas, & Cytryńska, 2014; Tsai, Loh, & Proft, 2016).

Many studies have reported that biological and chemical agents negatively affect metabolic and immune responses in insects (Sugeçti, Büyükgüzel, & Büyükgüzel, 2016; Kastamonuluoğlu, Büyükgüzel, & Büyükgüzel, 2020). Aspartate transferase (AST) and alanine transferase (ALT) activities significantly increase in insects, especially in cellular damage due to oxidative stress (Sertçelik, Sugeçti, Büyükgüzel, Necefoğlu, & Büyükgüzel, 2018). On the other hand, alkaline phosphatase (ALP) and amylase (AMYL) activities have important roles in regulating energy metabolism in insects against oxidative damage (Sugeçti & Büyükgüzel, 2018). The other metabolic enzyme, gamma glutamyl transferase (GGT), increases the availability of amino acids, mainly cysteine, for intracellular glutathione synthesis and plays an important role in maintaining non-enzymatic antioxidant defense against oxidative stress in organisms (Ndrepepa, Colleran, & Kastrati, 2018). In addition, oxidative damage to insect tissues is prevented by enzymatic such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) and non-enzymatic antioxidants such as amount of uric acid (UA), bilirubin (BIL) and albumin (ALB) (Mirończuk-Chodakowska, Witkowska, & Zujko, 2018; Alp & Coskun 2018). It is known that biochemical parameters such as total protein (TP) and urea are negatively affected as a result of oxidative damage in insects (Etebari, Bizhannia, Sorati, & Matindoost, 2007; Sertçelik et al, 2018).

In this study, the changes in the activity of metabolic enzymes such as AST, ALT, ALP, AMYL, GGT and amount of biochemical parameters such as ALB, BILD, BILT, TP, UA and UREA in hemolymph of *G. mellonella* larvae after infection with *K. oxytoca* were investigated.

MATERIALS AND METHODS

Insect culture

G. mellonella culture was obtained from the Insect Culture Laboratory in Biology department of Zonguldak Bülent Ecevit University. Insect culture was continued in an incubator set at 28 ± 2°C and 65 ± 5% humidity under laboratory conditions. Insects were reared in artificial diet until the seventh larval stage of *G. mellonella*. Artificial diet was composed of 420 g of bran, 150 ml of filtered honey, 150 ml of glycerol, 20 g of ground old dark honeycomb, and 30 ml of distilled water (Bronskill, 1961).

Experimental design

K. oxytoca (ATCC 8724) was obtained from local company. *K. oxytoca* was prepared in a brain heart solution according to the 0.5 McFarland standard. The solution (10µl) prepared according to this standard (about $1x10^7$ bacteria) was injected through the abdomen of the insect. Hemolymph was collected at 2, 4, 6 and 8 hours after injection. As the control group, only 10 µl brain heart solution was injected and hemolymph was collected 8 hours after the injection. Before the hemolymph was collected, the larvae were kept on ice for about 5 minutes and their surfaces were disinfected with ethyl alcohol. Samples were stored at -80°C until analyses. The experiments were repeated four times.

Biochemical analysis

The collected hemolymph was placed in homogenization buffer (w/v 1.15% potassium chloride (KCl), 25 mM dipotassium hydrogen phosphate (K_2HPO_4), 5 mM ethylen-diaminetetraacetic acid (EDTA), 2 mM phenylmethylsulphonil fluoride (PMSF), 2 mM dithiothreitol (DTT), pH 7.0). Extracts of hemolymph were prepared at 4°C by an ultrasonic homogenizer (Bandelin Sonoplus, HD2070, Germany). Then, solution was centrifuged at 10.000 g for 10 min at 4°C. The analyses were performed with the Roche Hitachi Cobas c501 instrument (Roche, Germany) using appropriate kits. ALT (Kit no: 46051001), AST (Kit no: 46004201), GGT (Kit no: 44686801), ALP (Kit No: 44211201), AMYL (Kit No: 45142401). ALB (Kit no: 44430501), BILD (Kit no: 46042501), BILT (Kit no: 43808801), UA (Kit no: 44535001), TP (Kit no: 44686201) and UREA (Kit no: 45141501).

Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyze data on cell damage indicators, metabolic enzymes non-enzymatic antioxidants and biochemical parameters of *G. mellonella*. Tukey's HSD test was used to determine the significance of the difference between the mean. All analyses were performed in SPSS v.15.0 (SPSS, Chicago, IL, USA). A probability level of 0.05 was used to check the significance of the difference between the averages. (SPSS, 2006).

RESULTS

Effect of *K. oxytoca* infection on metabolic enzymes activities in hemolymph of *G. mellonella* larvae

In the present study, AST activity statistically increased 8 hours after with *K. oxytoca* infection when compared the control group ($F_{4,15}$: 311.346, p<0.05). Similarly, ALT activity significantly increased in hemolymph of *G. mellonella* larvae at 8 hours after injection ($F_{4,15}$: 229.497, p<0.05). ALP activity decreased approximately 3-fold 8 hours after injection when compared to the control group ($F_{4,15}$: 16.545, p<0.05). AMYL activity significantly decreased from 12.75 U / L to 13.75 U / L 8 hours after with *K. oxytoca* infection ($F_{4,15}$: 227.019, p<0.05). Other metabolic enzyme GGT activity was statistically increased 8 hours after exposure to *K. oxytoca* ($F_{4,15}$: 24.468, p<0.05) (Table 1).

Table 1. Effect of *K. oxytoca* infection on metabolic enzymes activities in hemolymph of *G. mellonella* larvae.

Time	AST (U/L) (Mean*± S.E)†	ALT (U/L) (Mean*± S.E)†	ALP (U/L) (Mean*± S.E)†	AMYL (U/L) (Mean*± S.E)†	GGT (U/L) (Mean*± S.E)†
Control	59.25 ± 4.42a	9.50 ± 0.75a	9.75 ± 0.64a	12.75 ± 5.23a	4.27 ± 1.10a
2	68.50 ± 7.29a	30.50 ± 2.94b	4.85 ± 0.82b	12.50 ± 0.43a	2.55 ± 0.51a
4	58.50 ± 9.83a	33.50 ± 6.17b	5.00 ± 1.11b	95.00 ± 2.69b	2.75 ± 0.32a
6	55.00 ± 0.50a	72.50 ± 6.72c	1.15 ± 0.14c	29.75 ± 1.90c	2.00 ± 0.22a
8	333.01 ± 2.59b	217.01 ± 4.55d	3.30 ± 0.38bc	13.75 ± 0.86ad	14.9 ± 2.21b

* Mean of four replicates per treatment with 20 larvae per replicate

† Means within a column followed by the same lowercase letter are not significantly different (Tukey's HSD test: p > 0.05)

ϒ Control: 8 hours after brain-heart solution injection.

Effect of *K. oxytoca* infection on biochemical parameters in hemolymph of *G. mellonella* larvae

The amount of ALB decreased approximately 4-fold 8 hours after *K. oxytoca* infection ($F_{4,15}$: 58.160, p<0.05). The amount of BILD increased from 0.29 ± 0.02 mg / dL to 0.48 ± 0.05 mg / dL 8 hours after infection. However, this increase in the amount of BILD was not statistically significant ($F_{4,15}$: 3.153, p>0.05). The amount of BILT increased statistically 8 hours after *K. oxytoca* infection ($F_{4,15}$: 4.137, p<0.05). The amount of TP in hemolymph of *G. mellonella* larvae significantly decreased 8 hours after infection ($F_{4,15}$: 90.254, p<0.05). The amount of UA decreased from 0.58 ± 0.01 mg / dL to 0.26 ± 0.05 mg / dL 8 hours *K. oxytoca* infection ($F_{4,15}$: 6.852, p<0.05). The amount of UREA increased approximately 8-fold 8 hours after with *K. oxytoca* infection when compered the control group ($F_{4,15}$: 13.461, p<0.05). (Table 2).

DISCUSSION

In this study, the pathophysiological effects of the pathogen *K. oxytoca* on the invertebrate model organism *G. mellonella* were investigated. Biochemical parameters in

hemolymph of *G. mellonella* were affected negatively. In addition, changes in the amount of non-enzymatic antioxidants occurred in *G. mellonella* hemolymph due to the infection.

Table 2. Effect of *K. oxytoca* infection on biochemical parameters in hemolymph of *G. mellonella* larvae.

Time	ALB (g/dL) (Mean*± S.E)†	BILD (mg/dL) (Mean*± S.E)†	BILT (mg/dL) (Mean*± S.E)†	TP (g/dL) (Mean*± S.E)†	UA (mg/dL) (Mean*± S.E)†	UREA (mg/dL) (Mean*± S.E)†
Control$^{\gamma}$	2.15 ± 0.11a	0.29 ± 0.02a	0.39 ± 0.02a	6.72 ± 0.25a	0.58 ± 0.01a	2.55 ± 0.50a
2	1.55 ± 0.12b	0.41 ± 0.09a	0.62 ± 0.12b	4.71 ± 0.41b	0.64 ± 0.04a	16.20 ± 1.55b
4	1.85 ± 0.04ab	0.66 ± 0.15b	1.07 ± 0.25c	5.49 ± 0.08b	0.77 ± 0.15a	12.86 ± 3.22b
6	0.51 ± 0.05c	0.16 ± 0.03c	0.25 ± 0.03a	1.35 ± 0.02c	0.25 ± 0.02ab	5.05 ± 0.28a
8	0.50 ± 0.08c	0.48 ± 0.05a	0.71 ± 0.04b	1.42 ± 0.015c	0.26 ± 0.05ab	18.30 ± 1.19b

Mean of four replicates per treatment with 20 larvae per replicate

† Means within a column followed by the same lowercase letter are not significantly different (Tukey's HSD test: $p > 0.05$)

γ Control: 8 hours after brain-heart solution injection

Biological and chemical agents are known to disrupt the energy metabolism of insects, cause cell damage and alter the antioxidant system (İçen, Armutçu, Büyükgüzel, & Gürel, 2005; Sertçelik et al, 2018; Sugeçti & Büyükgüzel, 2018; Farahani, Bandani, Alizadeh, Goldansaz, & Whyard, 2020). In this study, it was determined that the cell damage indicators AST and ALT enzyme activities were increased in the hemolymph of *G. mellonella* larvae exposed to *K. oxytoca* for 8 hours. The reason for this increase may be cell damage due to the high virulence effect of the bacteria. In addition, ALP activity from metabolic enzymes was found to be decreased 8 hours after injection. The reason for this reduces may be increased oxidative damage due to the pathogenic effect of the bacteria. Studies have reported that ALP activity increases in insect tissues to regulate energy metabolism, which is disrupted by oxidative damage (Etebari et al, 2007; Sertçelik et al, 2018). In this study, it was determined that other metabolic GGT activity increased 8 hours after injection. GGT is an important metabolic enzyme that catalyzes the extracellular degradation of glutathione by hydrolyzing cysteine and γ glutamyl bond extracellularly. The amount of cysteine and glutamyl enters the cell and the synthesis of glutathione, an important antioxidant, increases (Sugeçti & Büyükgüzel, 2018). In this study, the increase in GGT activity may be physiological adaptation to increase the insect's antioxidant defense system. In another study, the physiological effects of an anthelmintic drug, oxfendazole, on the hemolymph of *G. mellonella* larvae were investigated. It has been determined that oxfendazole significantly increases the activity of cell damage indicators AST and ALT and the metabolic enzymes ALP, AMYL, CK and GGT (Sugeçti and Büyükgüzel 2018). In another study with similar results, the metal complex was injected into *G. mellonella* larvae and changes in metabolic enzymes activities were investigated. AST, ALT, LDH, CK, ALP and GGT activity increased significantly in the hemolymph of *G. mellonella* larvae 8 hours after injection of the metal complex (Sertçelik et al, 2018). In another study, transferase enzymes such as AST, ALT and GGT were increased in the hemolymph of great wax moth larvae due to *E.coli* infection (Sugeçti, 2021a).

Grizanova, Krytsyna, Surcova, & Dubovskiy (2019) reported that esterase activity was significantly reduced in *G. mellonella* midgut tissue due to *Bacillus thuringiensis* infection (Grizanova, Krytsyna, Surcova, & Dubovskiy, 2019). Wand, McCowen, Nugent, & Sutton (2013) reported that due to *K. pneumoniae* infection, LDH enzyme activity (cell damage indicator) increased in hemolymph of *G. mellonella* larvae and cell damage occurred (Wand, McCowen, Nugent, & Sutton, 2013). In another study, it was reported that cell damage was increased in *G. mellonella* larvae due to *K. pneumoniae* infection. It was determined that AST, ALT and LDH activities increased due to *K. pneumoniae* infection, and AMYL, ALP and CK enzyme activities also increased for the regulation of energy metabolism (Sugeçti, 2021b). These studies on the same insect show that biological and chemical agents increase the insect's metabolic enzymes and support our study.

Insects protect themselves against chemicals, especially insecticides and pathogenic bacteria infections with their antioxidant defense system (Dubovskii et al, 2010; Çelik & Sak, 2020). Antioxidant defense system is provided with enzymatic antioxidants such as CAT, SOD and GPx or non-enzymatic antioxidants such as ALB, UA and BIL. In this study, 8 hours after *K. oxytoca* injection, it was determined that the amount of non-enzymatic antioxidants BILT significantly increased. This increase may be due to adaptation to the oxidative damage caused by the pathogen. The amount of ALB and UA significantly decreased due to the infection. It has been reported in various studies that antioxidative levels increase in insects against the oxidative effects of pathogenic bacteria. In another study, it was reported that *G. mellonella* antioxidant effect increased due to *Bacillus thuringiensis* infection (Dubovskii et al, 2010). In another study, antioxidant enzymes such as SOD and GST levels were increased in *G. mellonella* larvae after infection with *B. thuringiensis* (Dubovskii, Olifirenko, & Glupov, 2005). Altuntaş & Duman (2017) reported that the antioxidant response in hemolymph of *G. mellonella* larvae to the entomopathogenic fungus *Purpureocillium lilacinus* infection altered as a physiological adaptation (Altuntaş & Duman 2017).

In this study, the amount of UREA in hemolymph of *G. mellonella* larvae significantly increased depending on the exposure time to the pathogen. The amount of TP decreased significantly due to *K. oxytoca* infection. It is known that especially the amount of protein increases in the regulation of energy metabolism against oxidative damage (Sertçelik et al, 2018; Çelik, Büyükgüzel, & Büyükgüzel, 2019). In this study, the reason for the decrease in the amount of TP may be damage due to infection. In particular, the increase in cell damage indicators AST and ALT activities supported this hypothesis. In a study, it has been reported that the chemical agent terbinafine (0.1 %) increased protein oxidation and causes protein damage in the midgut of *G. mellonella* larvae (Kastamonuluoğlu, Büyükgüzel, & Büyükgüzel, 2020). In another study, it was reported that the amount of TP increased in *G. mellonella* larvae exposed to different concentrations (0.003, 0.03 and 0.3%) of oxyclozanide (Çelik, Büyükgüzel, & Büyükgüzel, 2019). Etebari et al (2007) reported that the amount of TP and UREA in *Bombyx mori* L. (Lepidoptera: Bombycidae) larvae exposed to pyriproxyfen increased after 120 hours (Etebari et al, 2007). In another study, it was reported that biochemical parameters such as TP, UREA, cholesterol, ions and amount of non-enzymatic

antioxidants such as ALB, BIL and UA were altered due to 8 hours after *K. pneumoniae* infection in the hemolymph of last instar *G. mellonella* larvae (Sugeçti, 2021b).

The results obtained from this study showed that *G. mellonella* can be used as a model organism in the evaluation of the physiological effects of *K. oxytoca* infection on the host. As a result, this study showed that *G. mellonella* is an important experimental model in the investigation of biochemical interaction between pathogenic bacteria and host.

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