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Effects of *Lucilia sericata* Larval Secretion on *Echinococcus granulosus*

Lucilia sericata Larva Sıvısının Echinococcus granulosus Üzerine Etkileri

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ABSTRACT

Objective: *Echinococcus granulosus* causes echinococcosis when its larvae settle in various organs, especially the liver and lung, of humans and herbivorous animals such as sheep and cattle. Echinococcosis are endemic in Mediterranean countries including Türkiye, the Middle East and South Africa. Echinococcosis treatments are generally surgical excision or benzimidazoles. Maggot debridement therapy is a wound treatment with *Lucilia sericata* larvae. While the larvae consume the old-damaged tissues and bacteria in the wound site, the maggots secrete an antimicrobial fluid. In this study, we aimed to determine the effect of *Lucilia sericata* larval secretions on *Echinococcus granulosus* protoscoleces *in vitro*.
Methods: Various DNA damage markers were used to analyze the therapeutic potential of the larval secretions. For this purpose, protoscoleces were cultured and treated with different concentrations of larval secretions. Comet test was performed to determine DNA damage. Expression of *EgATM, EgRad9* and *EgTopo2a* genes was analyzed by quantitative real time polymerase chain reaction.
Results: The viability of the control group was 94% and the viability of the protoscoleces treated with larval secretions was 73%. Comet test showed that larval secretions caused DNA damage in protoscoleces. According to quantitative real time polymerase chain reaction results; 1:1 larval secretions increased *ATM* and *Rad9* gene expression 3.2-fold and *Topo2a* gene expression 2.2-fold compared to control groups ($p<0.05$).
Conclusion: These data showed that *in vitro* larval secretion induced DNA damage in *Echinococcus granulosus* protoscoleces and increased the expression of *EgATM, EgRad9* and *EgTopo2a* genes. However, further *in vitro* and *in vivo* studies are needed.
Keywords: *Echinococcus granulosus*, *Lucilia sericata*, DNA damage, Comet assay, gene expression

ÖZ

Amaç: *Echinococcus granulosus*'un larvaları, insanlarda ve koyun, sığır gibi otçul hayvanlarda özellikle karaciğer ve akciğer gibi çeşitli organlara yerleşerek ekinokokkozise neden olur. Ekinokokkozis, Türkiye dahil olmak üzere Akdeniz ülkeleri, Orta Doğu ve Güney Afrika'da endemiktir. Ekinokokkozis tedavileri genellikle cerrahi eksizyon veya benzimidazoller ile yapılmaktadır. *Lucilia sericata* larvaları ile uygulanan Maggot debridman tedavisi, bir yara tedavi yöntemidir. Larvalar, yara bölgesindeki eski ve hasar görmüş dokuları ve bakterileri elimine ederken, antimikrobiyal bir sıvı salgılar. Bu çalışmada, *Lucilia sericata* larval sekresyonlarının *Echinococcus granulosus* protoskoleksleri üzerindeki etkisini *in vitro* olarak belirlemeyi amaçladık.
Yöntemler: Larval sekresyonların tedavi potansiyelini analiz etmek için çeşitli DNA hasarı belirteçleri kullanıldı. Bu amaçla, protoskoleksler kültüre alındı ve farklı konsantrasyonlarda larval sekresyonlar ile muamele edildi.
Bulgular: DNA hasarını belirlemek için Comet testi uygulandı. *EgATM*, *EgRad9* ve *EgTopo2a* genlerinin ekspresyonu, kantitatif gerçek zamanlı polimeraz zincir reaksiyonu ile analiz edildi. Kontrol grubunda canlılık oranı %94 iken, larval sekresyonlar ile muamele edilen protoskolekslerde canlılık oranı %73 olarak belirlendi. Comet testi, larval sekresyonların protoskolekslerde DNA hasarına yol açtığını gösterdi. Kantitatif gerçek zamanlı polimeraz zincir reaksiyonu sonuçlarına göre; 1:1 oranında larval sekresyon, *ATM* ve *Rad9* gen ekspresyonunu kontrol gruplarına kıyasla 3,2 kat, *Topo2a* gen ekspresyonunu ise 2,2 kat artırdı ($p<0,05$).
Sonuç: Bu veriler, *in vitro* olarak larval sekresyonların *Echinococcus granulosus* protoskolekslerinde DNA hasarını indüklediğini ve *EgATM*, *EgRad9* ve *EgTopo2a* genlerinin ekspresyonunu artırdığını göstermiştir. Ancak, bu sonuçların desteklenmesi için daha fazla *in vitro* ve *in vivo* çalışmalara ihtiyaç vardır.
Anahtar Kelimeler: *Echinococcus granulosus*, *Lucilia sericata*, DNA hasarı, Comet testi, gen ekspresyonu

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Echinococcus granulosus is a parasite that causes echinococcosis when its larvae settle in various organs and tissues, especially the liver and lungs, of humans and herbivorous animals such as sheep and cattle (1, 2). Echinococcosis is found on every continent except Antarctica. It is endemic in tropical and subtropical regions, especially where livestock farming is widespread, such as Mediterranean countries, Türkiye, the Middle East, South America, New Zealand, and South Africa. In endemic areas, incidence rates can be 50/100.000 or more (3).

The disease is treated by surgery, punction-aspiration-injection-reaspiration method or chemotherapy. In chemotherapy, benzimidazoles are used, particularly albendazole (ABZ). ABZ is a benzimidazole derivative, and the active metabolite responsible for its anthelmintic effects is ABZ sulfoxide. Systemic absorption of the drug is low; therefore, high doses are required to treat the disease, which makes it difficult for the patient to tolerate the drug (4). Additionally, teratogenic and embryotoxic effects of the drug have been reported in experimental animal studies (5). ABZ is also used as a protoscolocidal agent, they are used during surgical and percutaneous treatment of echinococcosis against the risk of secondary cysts or recurrence (6). For this purpose, various chemical compounds and plant extracts have been frequently tested in recent years (3,7).

The use of *Lucilia sericata* larvae in wound treatment is known as maggot therapy. They provide wound healing by debridement and disinfection and by inducing the secretion of wound-healing stimulants (8). In addition, excretions/secretions (ES) secreted by larvae also show antimicrobial activity. Studies have proven that larval ES contain various proteinases, DNases and antimicrobial substances such as lusifensin and lusimycin (9-12). The effect on many Gram-positive and some Gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus,* various fungi and parasites of the genus *Leishmania* has been studied (8,13). Studies on cancer cell lines have shown that larval ES limits tumor growth and metastasis and causes increased expression of apoptotic genes, consequently affecting eukaryotic cells at the molecular level (14,15).

Cellular DNA undergoes physical and/or chemical changes due to various factors such as free oxygen radicals, intracellular stresses, ultraviolet radiation and ionizing radiation (X-ray), resulting in DNA damages (single or double-strand breaks, base changes, base dimers and/or nucleotide losses or gains). DNA damage can be detected by various methods, such as Comet and TUNEL (16). In addition, gene expression is considered to be the best means of identifying DNA damage, and quantitative real time-polymerase chain reaction (qRT-PCR) has been the most widely used method for this. Gene regions such as *ATM, ATR, Rad9, p53* and *Topo2* have been used in various studies for this purpose (17-19).

In this study, we aimed to determine the effect of Lucilia sericata larval secretions on Echinococcus granulosus protoscoleces *in vitro* and whether the larval secretions can be used as treatment option. Various DNA damage markers were used to analyze the therapeutic potential of the larval secretions.

METHODS

Collection of Cyst Fluid and Determination of Viability

This study was conducted with the approval of Trakya University Faculty of Medicine Scientific Research Ethics Committee (no: TÜTF-BAEK-2021/368, date: 20.09.2021). The study does not require informed consent.

One hydatid cyst material used in our study was obtained fresh from newly slaughtered sheep at Edirne Meat and Meat Products Integrated Facility, Industry and Commerce Company Slaughterhouse. Cyst fluid was obtained based on the method of Smyth and Davies (20). The cyst contents were transferred to sterile falcon tubes and kept at room temperature for 30 min to allow the protoscoleces to settle. At the end of the time, the upper liquid was discarded, and the cyst material at the bottom of the tube was used for viability determination with 0.1% eosin.

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Protoscolex Culture

The culture of *E. granulosus* protoscoleces was performed by modifying the method of Smyth and Davies (20). Briefly, the cyst material was washed 4 times with Hank's Balanced Salt Solution, and after each washing, protoscoleces were allowed to settle at room temperature for 30 min. In the last step, after waiting for 30 minutes, the upper liquid was discarded, replaced with RPMI medium (maintenance medium; RPMI 1640 with 20% FBS, 1% penicillin-streptomycin solution), and washed for 10 minutes; the medium was renewed at the end of the time. This mixture was distributed into 5 flasks, with the number of protoscolices per mL being equal to each flask (approximately 1000 protoscolices per mL). RPMI medium was added to each flask to a final volume of 5 mL, and the flasks were incubated in a 37 °C CO_2 incubator for 24-48 hours.

Preparation and Application of Larval Secretion

Lucilia sericata larvae were obtained from the insectarium of İstanbul University-Cerrahpaşa Traditional and Complementary Medicine Research and Development Center. Larval secretion was prepared according to the method of Akbas et al. (21). Briefly, second and third-stage approximately 2000 sterile larvae were placed in a sterile beaker, and 5 mL of distilled water was added to them. 5 mL of distilled water was added at one-hour intervals for 5 hours, and the larvae were allowed to release their secretions into the water. After all, 30 mL of larval ES collected. After the process, the liquid was centrifuged at 1.500 rpm for 10 min, and the supernatant was separated for use. After this liquid was sterilized by filtration, various dilutions were prepared, and 5 mL each of 1:1, 1:2, and 1:4 dilutions were added to the flasks. ABZ solution at a concentration of 20 µg/mL was added to the fourth flask as a positive control. Nothing was added to the fifth flask and used as a negative control. After treatment, the flasks were incubated at 37 °C for 5 hours for Comet test and microscopic examination and 24-48 hours for qRT-PCR.

Comet Test

Comet test was performed by modifying the method of Singh et al. (22). Briefly, 5 hours after the application, the samples

taken from the flasks were mixed with 0.75% low melting point agarose and spread on slides previously coated with 1.5% normal melting point agarose. The slides were first kept in a lysis solution for 2 hours and then in an alkaline electrophoresis buffer for 1 hour. At the end of the time, electrophoresis was started by setting the current to 300 mA, voltage to 24 V and time to 1 hour. After electrophoresis, the slides were stained with ethidium bromide and visualized under a fluorescence microscope (Olympus BX51, 40x magnification). The results of the Comet test were evaluated from Grade 0 to +4 with a visual scoring diagram from Noroozi et al. (23); Grade 0 no damage (<5%), Grade 1 low damage (5-25%), Grade 2 medium damage (25-45%), Grade 3 high damage (45-70%) and Grade 4 very high damage (>70%). Collins method was used to calculate the total score (24): 50 comets are scored each flask (total score was between 0-200), and each comet assigned a value of 0 to 4 according to its class and total score calculated.

qRT-PCR

For the detection of *EgATM* (*EGR_01160*), *EgRad9 (EGR_01432)* and *EgTopo2a* (*EGR_04559*) gene expressions of *E. granulosus* in samples taken from treated flasks, qRT-PCR was performed based on the method of Higuchi et al. (25). Each experiment was carried out in triplicate. RNA isolation was performed according to the recommendations of the kit manufacturer (QuickEx Total RNA Extraction Kit, Nucleogene, Türkiye). Then, the cDNA of each group was synthesized using the isolated RNAs according to the instructions of the kit manufacturer (cDNA Synthesis Kit with RNase Inh.-High Capacity, A.B.T., Türkiye). Gene-specific primer sequences were designed (Table 1) and these were synthesized commercially (Genesuz, Türkiye). Applied Biosystems Quantistudio 6 Flex real-time PCR instrument (Thermo Fisher Scientific, USA) was set up according to kit instructions (qPCR Probe Master Mix-2X kit, Nucleogene, Türkiye) and qRT-PCR was performed. As a result of the process, gene expressions of EgATM, EgRad9 and EgTopo2a of *E. granulosus* were calculated by the ΔΔCt method, based on Ct values (26).

Statistical Analysis

GraphPad Prism 9 statistical software was used to perform statistical analyses. Comparisons between two groups were analyzed by using t-test, and the comparison between multiple groups was analyzed by using One-Way ANOVA. $P<0.05$ indicated that the difference was statistically significant.

RESULTS

Implementation Results

The viability rate of the hydatid cyst material, in which protoscolex was detected on direct microscopic examination and included in the study, was 95%.

When the culture flasks were applied and microscopically examined 5 hours later, the viability rates were 73% for larval ES 1:1, 80% for larval ES 1:2, 86% for larval ES 1:4, 70% for ABZ and 94% for control.

Comet Test

According to the results of the Comet test performed 5 hours after the application, total scores were 2 for control, 120 for larval ES 1:1, 90 for larval ES 1:2, 63 for larval ES 1:4 and 127 for ABZ. Grade 4 damage was observed mainly in Larval ES 1:1 application (36%), Grade 0 damage was observed mainly in Larval ES 1:2 and Larval ES 1:4 applications (31.3% and 37.3%, respectively), and Grade 3 damage was observed mainly in ABZ application (36.9%). In the control group, the majority was Grade 0 damage (96.8%), and no Grade 2, Grade 3 and Grade 4 damage was observed. The larval ES 1:1 group had the most Grade 4 damage (36%).

qRT-PCR

EgATM gene expression increased 3.2, 2.6, 1.8 fold in Larval ES dilutions 1:1, 1:2, 1:4 respectively and 4.5-fold in ABZ treated protoscoleces. Gene expression in protoscoleces from all treatment groups was statistically significant compared to the gene expression in control group protoscoleces ($p<0.05$).

EgRad9 gene expression increased 3.2, 1.4, 1.8 fold in Larval ES dilutions 1:1, 1:2, 1:4 respectively and 13.5-fold in ABZ treated protoscoleces. Gene expression in protoscoleces from all treatment groups was statistically significant compared to gene expression in control group protoscoleces ($p<0.05$).

EgTopo2a gene expression increased 2.2, 1.3, 1.6 fold in Larval ES dilutions 1:1, 1:2, 1:4 respectively and 12.9-fold in ABZ treated protoscoleces. Gene expression in protoscoleces from all treatment groups was statistically significant compared to the gene expression in control group protoscoleces ($p<0.05$). All gene expression increases are given in the Table 2.

DISCUSSION

E. granulosus, which causes hydatid cyst disease, is a prevalent parasite worldwide, threatening public health and causing economic losses by causing diseases in humans and animals

Table 1. Gene-specific primer sequences

Gene/gene ID	Primer sequences
EgATM 36336875	Forward primer: 5'- GTT CCT ACA GTC CAT CCT AAT- 3' Reverse primer: 5'- CTC CAT CAA GCC AGC ATT- 3';
EgRad9 36337147	Forward primer: 5'- ACC TGT CAA ATC GTG ATG TCG T- 3' Reverse primer: 5'- TCA CTT CAG TTT GAC TTG GCC T- 3';
EgTopo2a 36340274	Forward primer: 5'-CTT ACC ACT GCG GCC AAG TC- 3' Reverse primer: 5'- GCC ACC AGG GGT ATC AGA GT- 3'
Egβ-actin 36344784	Forward primer: 5'-GCG ATG TAT GTA GCT ATC CAG GCA GTG CTC TCG CT- 3' Reverse primer: 5'-CAA TCC TCC AGA CAG AGT ATT TGC GTT CCG GAG GA- 3'

Table 2. Gene expression increases

Gene/gene expression increase (fold)	Larval ES 1/1	Larval ES 1/2	Larval ES 1/4	ABZ
EgATM	3.2	2.6	1.8	4.5
EgRad9	3.2	1.4	1.8	13.5
EgTopo2a	2.2	1.3	1.6	12.9

ABZ: Albendazole

(6). Hydatid cysts are an important health problem in Türkiye, and cases are reported from all over the country. According to Ministry of Health data, the number of cases has increased in recent years; while the number of cases was 408 in 2008, the number of cases was reported as 1.867 at the end of 2019 (27). The estimated incidence rate of hydatid cyst disease in Türkiye is 0,8-2,0/100.000 (28). The disease is usually asymptomatic for years, and when it reaches a specific size, symptoms appear due to pressure on adjacent organs. The disease is most commonly recognized when radiologic tests are performed for other purposes (2,3).

During surgical and percutaneous treatment, protoscolicidal agents are used by many clinicians against secondary cyst formation or recurrence due to cyst rupture. Especially in the last 50 years, a wide range of protoscolicidal agents such as chemical compounds, plant extracts or natural compounds have been studied, and the search for effective alternative treatments for *E. granulosus* has increased. ABZ is frequently used as a protoscolicidal agent during surgical and percutaneous procedures (7). Apart from ABZ, harmin (19), thymol (29) and carvacrol (30) were the most studied chemical compounds as protoscolicidal agents *in vitro*. Besides, extracts of various plants such as *Cinnamomum zeylanicum* (31), *Curcuma longa* (32) and *Nigella sativa* (33), which were studied as protoscolicidal agents, showed a 100% killing effect on protoscoleces depending on dose and time. As a result of our study, the viability rate of protoscoleces 5 hours after treatment was 73% for larval ES 1:1 and 70% for ABZ. Since ABZ is frequently used as a protoscolicidal agent, we used ABZ as a positive control in our study. However, we attribute the lower lethality of the application compared to the literature to its short incubation period.

Lucilia sericata larvae are used in maggot therapy, which is therapy for various ulcers such as diabetic foot, ischemic skin, and decubitus ulcers (8,34). Studies have revealed the antimicrobial activity of secretions obtained from larvae (11,12,35-39). According to the results of studies in the literature, Larval ES showed bactericidal and/or bacteriostatic effects against various Gram-positive and Gram-negative bacteria (37-39). Cerovský et al. (11) isolated lucifensin from larval ES and identified it as a key component of the antibacterial effect. Although there are fewer studies on the effect of larval ES on fungi, Pöppel et al. (12) succeeded in isolating lucimycin, the compound responsible for the antifungal effect. As a result of studies on fungi, the antifungal activity of larval ES against various opportunistic pathogenic fungi has also been proven (35,36).

Besides of these studies, the antiparasitic effect of larval ES on protozoan parasites of the genus *Leishmania* has been also proven (13,40,41). As a result of the studies first conducted by Polat et al. (13), many researchers have focused on the anti-leishmanial effects of larval ES. However, no other parasite other than *Leishmania* spp. has been studied in the literature.

In our study, *in vitro* protoscolicidal effects of larval ES were examined for the first time, and viability was found to be significantly reduced compared to the control. In addition, the protoscolicidal activity of larval ES was also determined by showing DNA damage by the Comet test and changes in gene expression levels by qRT-PCR.

Lu et al. (19) and Gong et al. (18) used the Comet test in their study in which they evaluated the antiparasitic activity and DNA damage mechanisms of harmin in *Peganum harmala* seeds against *E. granulosus*. The results of these studies showed that the nuclei of the protoscoleces in the control group were compact without tails, while the nuclei of the protoscoleces in the harmin-treated group formed tails. As a result of our study in which the *in vitro* activity of larval ES on *E. granulosus* protoscoleces was examined, it was found that larval ES caused damage in protoscolex DNA, similar to the data in the literature. In terms of total DNA damage score, ABZ (127/200) and larval ES 1:1 (120/200) caused the most damage. Ramírez et al. (42) and Oztas et al. (43) have shown the DNA damaging effect of ABZ, in our study, ABZ caused DNA damage in the protoscoleces, and the results of our study overlap with these studies. Average damage grade of larval ES 1:1 was 4 (36%), while the average damage grade of the control group was 0 (96.8%). Moreover, the group with the highest damage grade 4 was the larval ES 1:1 group, with 36%, followed by the larval ES 1:2 group, with 22.9% and the ABZ group, with 16%. In this context, larval ES causes more severe DNA damage depending on the concentration.

In studies with *E. granulosus*, genes encoding various proteins have been used to determine the gene expression of a chemical substance in the parasite (17-19,44). The *ATM, Rad9*, and *Topo2a* genes that we used in our study are genes that are involved in the identification of DNA damage, especially at cell cycle checkpoints. The results of our study showed that 1:1 dilution of larval ES increased the expression of *EgATM* and *EgRad9* genes 3.2-fold and *EgTopo2a* gene expression 2.2-fold compared to the control. Cabrera et al. (17) showed that the expression of *Rad9* increased in the oxidative damage of *E. granulosus* protoscolex DNAs. Gong et al. (18) and Lu et al. (19) investigated the effects of harmin on *E. granulosus* and reported that the expression levels of *EgATM, EgP53, EgRad54* and *EgTopo2a* genes increased in harmin-treated protoscoleces. These results are consistent with the results of our study and indicate that larval ES induces *ATM-Rad9-Topo2a* signaling pathways by causing DNA damage.

In the present study, the effects of *L. sericata* larval ES on *E. granulosus* protoscoleces *in vitro* were reported. As a result of the study, larval ES caused DNA damage by showing a protoscolicidal effect. It increased the expression of *ATM-Rad9-Topo2a* genes involved in the cell cycle. In conclusion, the efficacy of larval ES against *E. granulosus* has been determined, and further *in vitro* and *in vivo* studies are needed.

*Ethics

Ethics Committee Approval: This study was conducted with the approval of Trakya University Faculty of Medicine Scientific Research Ethics Committee (no: TÜTF-BAEK-2021/368, date: 20.09.2021).

Informed Consent: The study does not require informed consent.

Footnotes

*Authorship Contributions

Surgical and Medical Practices: F.İ.A., Concept: E.P., N.Ş., Design: E.P., N.Ş., Data Collection or Processing: F.İ.A., K.T., Analysis or Interpretation: K.T., E.P., N.Ş., Literature Search: F.İ.A., N.Ş., Writing: F.İ.A., N.Ş.

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