Slovenian Veterinary Research

Slovenski veterinarski zbornik

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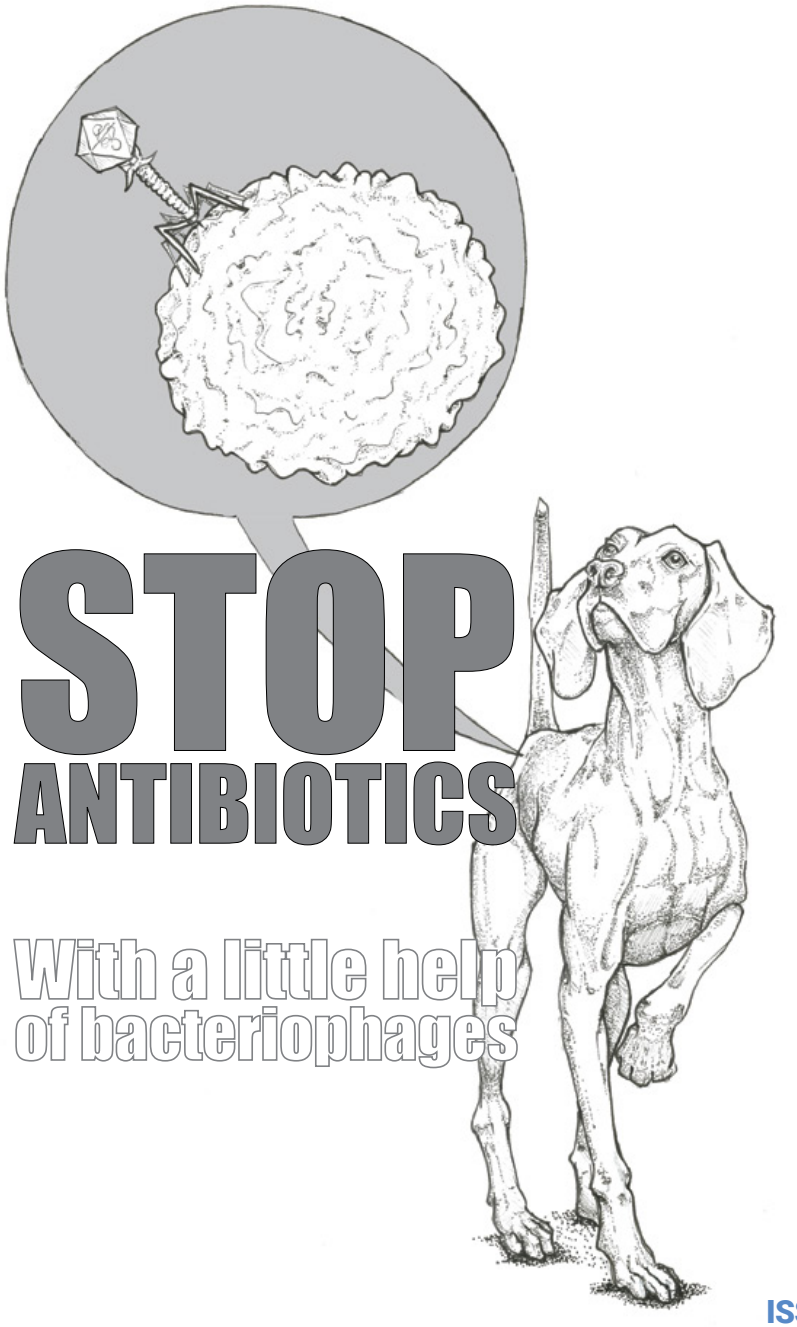

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Editorial

Rediscovering Phage Therapy: Promising Approach for Combating Antimicrobial Resistance

Obujanje fagne terapije: obetaven pristop za boj s protimikrobno odpornostjo

Key words

antimicrobial resistance;
bacteriophage;
phage therapy

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Antimicrobial resistance (AMR) is a concerning public health threat which affects human and animal health as well as the environment. The rapid spread of bacterial strains resistant to clinically used antibacterials necessitates the exploration and utilization of different treatment options. Phage therapy, the use of bacterial viruses – bacteriophages – to treat bacterial infections, has gained renewed interest as an aid in addressing AMR. As we outline in this editorial, the introduction and widespread use of phage therapy in human and veterinary medicine faces regulatory challenges. However, the recent adoption of new guidelines and other regulatory developments in this area will facilitate the progress of phage therapy.

Antimicrobial resistance was responsible for 1.2 million deaths globally in 2019 (1). The most pessimistic projections estimate 10 million deaths per year in 2050, meaning AMR will be more deadly than cancer, if no action is taken (2,3). Bacteria carrying antibiotic resistance genes can be transmitted between humans, animals, plants and the environment. Therefore, the fight against AMR requires unified and multidisciplinary action (OneHealth approach). Although livestock farming accounts for up to 80 % of total antibiotic consumption, infections in companion animals are predominantly treated with human antibiotics. As a result, pets become reservoirs for zoonotic bacterial species carrying resistance

Protimikrobna odpornost (AMR) predstavlja resno grožnjo javnemu zdravju in vpliva tako na zdravje ljudi in živali kot na okolje. Hitro širjenje bakterijskih sevov, ki so odporni na klinično uporabljane protibakterijske učinkovine, zahteva raziskovanje in uporabo drugačnih možnosti zdravljenja. Fagna terapija, uporaba bakterijskih virusov – bakteriofagov – za zdravljenje bakterijskih okužb, je pridobila ponovno zanimanje kot pomoč pri reševanju problema protimikrobne odpornosti. Kot opisujemo v tem uvodniku, se uvedba in široka uporaba fagne terapije v humani in veterinarski medicini sooča z regulatornimi izzivi, vendar pa bo nedavno sprejetje novih smernic in razvoj drugih regulatornih predpisov na tem področju olajšal napredek zdravljenja s fagi.

Protimikrobna odpornost je leta 2019 povzročila 1,2 milijona smrti po celem svetu (1). Najbolj pesimistične napovedi ocenjujejo, da bi lahko, če ne bomo ukrepali, leta 2050 zaradi protimikrobne odpornosti umrlo 10 milijonov ljudi na leto, kar pomeni, da bo povzročila več smrti kot rak (2,3). Bakterije, ki nosijo gene za odpornost na protibakterijske učinkovine, se lahko prenašajo med ljudmi, živalmi, rastlinami in okoljem, zato boj proti protimikrobni odpornosti zahteva enotno in multidisciplinarno delovanje (pristop OneHealth). Čeprav se v živinoreji porabi do 80 % celotne porabe protibakterijskih učinkovin, se okužbe domačih živali večinoma zdravijo

genes of clinical importance to humans (4–6). As outlined in the review article, published in this issue, canine skin infections are a frequent complication in small animal medicine, where the prevalence of antimicrobialresistant bacteria is increasing, necessitating additional treatment approaches such as vaccines and phage therapy (7).

Phage therapy is a treatment option based on natural predators of bacteria, the bacteriophages (phages). Their antibacterial mechanism of action differs from that of antibiotics and enables an effect on antimicrobial-resistant bacteria without causing cross-resistance. In addition, phages have a narrow host range, which prevents undesirable effects on the microbiota. They are non-toxic, can of self-replicate at the site of infection and have anti-biofilm activity (8,9). Their therapeutic potential was recognized as early as 1919, when Felix d'Herelle used phages to treat dysentery (10). The clinical success of these initial trials encouraged further applications, from the treatment of staphylococcal skin diseases to cholera and bubonic plague (10). Despite the promising initial results, early controversies in phage clinical research, inadequate understanding of phage biology and political reasons topped with the discovery of antibiotics pushed phage therapy into obscurity in Western countries (11). With the rise of AMR, phages are regaining the importance and attention of scientific and business community investing considerable efforts into development of novel phage-based antibacterial therapeutics. The poorly defined regulatory status of phages has been a bottleneck. However, the European Medicines Agency (EMA) has recently published Guideline on quality, safety and efficacy of veterinary medicinal products specifically designed for phage therapy, providing a regulatory framework for the use of phage therapy products in veterinary medicine. Phage therapies are classified as novel therapies by Regulation (EU) 2019/6 and require a centralized marketing authorization procedure involving clinical trials (12). Although the guidelines offer some flexibility, e.g. multiphage compositions can be regularly updated or reconditioned due to the narrow host range and development of resistance, the path to product authorization is expected to be long and expensive. Marketing authorization is not required for prescription medicinal products prepared in a pharmacy, so called magistral formulae, which are manufactured according to European Pharmacopoeia (Ph. Eur.). In July 2024, the European Pharmacopoeia Commission (EPC) will adopt a new general chapter in the European Pharmacopoeia, which will cover phage therapy medicinal products. The chapter provides requirements for the production and quality control of phage therapy products for human and veterinary use and is already pre-published on the European Directorate for the Quality of Medicines & HealthCare (EDQM) website (13). This document will facilitate the use of phage magistral preparations throughout the EU allowing more personalized approach, as was already in practice in Belgium for human use (14). The regulatory guidelines make it clear, that not all phages are suitable for phage therapy. The most important phage characteristic for therapy is their lytic lifestyle, which ensures the killing of bacteria at the end of their replication cycle. In addition, therapeutic

z učinkovinami razvitimi za humano uporabo. Posledično postajajo hišni ljubljenčki rezervoarji za zoonotske bakterijske vrste, ki prenašajo gene za odpornost, ki so klinično pomembni za ljudi (4–6). Kot je poudarjeno v preglednem članku, objavljenem v tej izdaji, so pasje okužbe kože pogost zaplet v medicini malih živali, kjer narašča razširjenost odpornih bakterij, zaradi česar so za zdravljenje potrebni dodatni pristopi, kot so cepiva in fagna terapija (7).

Fagna terapija je možnost zdravljenja, ki temelji na naravnih zajedavcih bakterij, bakteriofagih (fagih). Njihov protibakterijski mehanizem delovanja se razlikuje od mehanizma delovanja protibakterijskih učinkovin in omogoča delovanje na odporne bakterije brez povzročitve navzkrižne odpornosti. Poleg tega imajo fagi ozek nabor gostiteljev, kar preprečuje neželene učinke na mikrobioto, niso toksični, lahko se samo-podvojujejo na mestu okužbe in delujejo proti bakterijskim biofilmom (8,9). Njihov terapevtski potencial je bil prepoznan že leta 1919, ko je Felix d'Herelle uporabil fage za zdravljenje dizenterije (10). Klinični uspeh začetnih preskušanj je spodbudil nadaljnjo uporabo, od zdravljenja stafilokoknih kožnih bolezni do kolere in bubonske kuge (10). Kljub obetavnim začetnim rezultatom so zgodnje polemike v kliničnih raziskavah fagov, neustrezno razumevanje biologije fagov, politični razlogi, poleg ega pa še odkritje protibakterijskih učinkovin, potisnili fagno terapijo v zahodnih državah v pozabo (11). Z vzponom protimikrobne odpornosti fagi ponovno pridobivajo pomembnost in pozornost znanstvene skupnosti in industrije, ki vlaga veliko truda v razvoj novih protibakterijskih terapij na osnovi fagov. Težavo je dolgo predstavljal slabo definiran egulatorni status fagov, vendar pa je Evropska agencija za zdravila (EMA) nedavno objavila Smernice o kakovosti, varnosti in učinkovitosti zdravil za uporabo v veterinarski medicini, posebej zasnovanih za terapijo s fagi, ki zagotavljajo regulatorni okvir za uporabo izdelkov za agno terapijo v veterinarski medicini. Fagne terapije so z Uredbo (EU) 2019/6 razvrščene kot nove terapije in zahtevajo centraliziran postopek pridobitve dovoljenja za promet, ki vključuje klinična preskušanja (12). Čeprav smernice omogočajo nekaj prilagodljivosti, npr. fagne koktejle je mogoče redno posodabljati ali obnavljati zaradi ozkega obsega gostiteljev in razvoja odpornosti, se pričakuje, da bo pot do odobritve izdelka dolga in draga. Za zdravila na recept, tako imenovana magistralna zdravila, ki so pripravljena v lekarni, in so izdelana v skladu z Evropsko farmakopejo (Ph. Eur.), registracija ni potrebna. Julija 2024 bo Komisija za Ph. Eur. sprejela novo splošno poglavje Evropske farmakopeje, ki bo zajemalo zdravila za fagno terapijo. Poglavje določa zahteve za proizvodnjo in nadzor kakovosti fagnih terapevtskih izdelkov za humano in veterinarsko uporabo, osnutek je na voljo v predogled na spletni strani Evropskega direktorata za kakovost zdravil (EDQM) (13). Ta dokument bo olajšal uporabo fagnih magistralnih pripravkov po vsej EU, kar bo omogočilo personaliziran pristop, kot je za humano uporabo že v praksi v Belgiji (14). Regulatorne smernice jasno navajajo, da vsi fagi niso primerni za terapijo. Najpomembnejša značilnost terapevtskih fagov je njihov litični življenjski slog, ki zagotavlja uničenje bakterij na koncu

phages should not contain genetic elements encoding toxins, virulence factors, antibiotic resistance and lysogeny-related genes (15).

Several classes of phage-based products are currently under development, either for use in humans, veterinary medicine or the environment. Phages isolated from environmental sources, are referred to as natural phages. Although they may be suitable for therapeutic applications, they cannot be readily patented, which makes them less attractive for drug development. Nevertheless, natural phages are convenient for use in magistral preparations and there are several research groups, collecting natural phages in biobanks available for the most critical clinical cases (16). Genetically engineered phages are modified to increase their efficacy (improving host range or delaying the emergence of phage-resistant bacteria), or to improve their pharmacokinetics (17). Although the extent of the clinical superiority of genetically engineered phages is currently unclear, patentability of these phages attracts investors and enables the development of such products. Another class of phage-based products are phage proteins with antibacterial activity, such as endolysins and depolymerases, as reviewed in (18). As these are proteins by nature, they are subject to different regulatory requirements and there are fewer intellectual property protection issues than with natural phages.

Phage therapy and phage-based products are an important additional treatment option for bacterial infections, which will never completely replace antibiotics. However, with increasing AMR and associated problems, it is crucial to invest in phage research and development to expand the toolbox of weapons against deadly bacterial infections.

njihovega replikacijskega cikla. Poleg tega terapevtski fagi ne smejo vsebovati genetskih elementov, ki kodirajo toksine, virulentne dejavnike, odpornost na antibiotike in genov povezanih z lizogenim ciklom (15).

Trenutno je v razvoju več razredov izdelkov na osnovi fagov, bodisi za uporabo pri ljudeh, v veterinarski medicini bodisi okolju. Fage, izolirane iz okolja, imenujemo naravni fagi. Čeprav so lahko primerni za terapevtske aplikacije, jih ni mogoče enostavno patentirati, zaradi česar so manj privlačni za razvoj zdravil. Kljub temu so primerni za uporabo v magistralnih pripravkih in obstaja več raziskovalnih skupin po svetu, ki naravne fage zbirajo v biobanke s čimer omogočajo fage za zdravljenje najbolj kritičnih kliničnih primerov (16). Gensko spremenjeni fagi so spremenjeni z namenom povečanja njihove učinkovitosti (razširitev obsega gostiteljev ali odložitev pojava na fage odpornih bakterij) ali za izboljšanje njihove farmakokinetike (17). Čeprav obseg klinične superiornosti gensko spremenjenih fagov trenutno še ni jasen, možnost patentne zaščite takih produktov privablja vlagatelje in omogoča sredstva za razvoj takšnih izdelkov. Drug razred izdelkov na osnovi fagov so fagni proteini s protibakterijskim delovanjem, kot so endolizini in depolimeraze, kot je opisano v (18). Ker so to po naravi beljakovine, zanje veljajo drugačne regulatorne zahteve, poleg tega je v teh primerih manj težav z zaščito intelektualne lastnine kot pri naravnih fagih.

Fagna terapija in izdelki na osnovi fagov so pomembna dodatna možnost zdravljenja bakterijskih okužb, ki ne bodo nikoli popolnoma nadomestili protibakterijskih učinkovin. Vendar pa je zaradi naraščajoče protimikrobne odpornosti in s tem povezanih težav ključno vlagati v raziskave in razvoj fagov, da bi razširili nabor orožja proti smrtonosnim bakterijskim okužbam.

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Human Herpesvirus Epstein-Barr (EBV) and Its Porcine Homologs Unveil the Conserved Mechanism of Receptor Endocytosis: New Insights Into Viral Immune Evasion and Antiviral Therapy Potential?

Humani herpesvirus Epstein-Barr (EBV) in njegovi prašičji homologi razkrivajo ohranjeni mehanizem receptorske endocitoze: nov vpogled v virusno izmikanje imunskemu sistemu in potencialne protivirusne terapije?

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Over the past two years, the University of Ljubljana and the Republic of Slovenia's public agency, the Slovenian Research and Innovation Service (ARIS), have acknowledged and celebrated several exceptional accomplishments in viral receptor research. These achievements are considered among the finest by both the University of Ljubljana and ARIS. The significance of these achievements lies in the research findings, which indicate that the EBV-BILF1 receptor encoded by the Epstein-Barr virus (EBV) could become a promising new drug target for EBV. Additionally, the research suggests pigs represent a great model for further investigations (1—4).

Epstein-Barr virus (EBV), also known as human herpesvirus 4 (HHV4), is found in about 95% of the world's adult population and causes infectious mononucleosis (5). EBV is also an oncovirus and is associated with various types of lymphoma and other cancers. In patients with compromised immune system following solid organ transplants (SOT) and hematopoietic stem cell transplants (HSCT) EBV infection is considered as a driving factor for the development of post-transplant lymphoproliferative disorder (PTLD), which leads to various types of tumours with a high risk of fatal outcome (6,7).

Univerza v Ljubljani in Javna agencija za znanstvenoraziskovalno in inovacijsko dejavnost Republike Slovenije (ARIS) sta v preteklih dveh letih prepoznali in počastili več izjemnih dosežkov na področju raziskovanja virusnih receptorjev. Ti dosežki – tako na Univerzi v Ljubljani kot na ARIS-u – sodijo med najboljše. Njihov pomen izhaja iz raziskav, ki kažejo na to, da bi se lahko receptor EBV-BILF1, kodiran na virusu Epstein-Barr (EBV), uporabljal kot obetavna nova tarča za zdravljenje EBV. Poleg tega raziskava predlaga uporabo prašičev kot modela za nadaljnje preiskave (1—4).

Virus Epstein-Barr (EBV), poznan tudi kot humani herpesvirus 4 (HHV4), je prisoten pri približno 95 odstotkih odraslega svetovnega prebivalstva in povzroča infekcijsko mononukleozo (5). EBV je tudi onkovirus in je povezan z različnimi vrstami limfoma in drugimi vrstami raka. Pri bolnikih z ogroženim imunskim sistemom po presaditvi trdnih organov (SOT) in presaditvi hematopoetskih matičnih celic (HSCT) okužba z EBV šteje za vodilni dejavnik za razvoj posttransplantacijske limfoproliferativne motnje (PTLD), ki vodi do različnih vrst tumorjev z visokim tveganjem za usoden izid (6, 7).

To achieve such severe complications many herpesviruses, including EBV, human cytomegalovirus (HCMV) and Kaposi's sarcoma-associated virus (KSHV), have developed various strategies to infect and persist in the host for a lifetime (8,9). One of these strategies is the expression of viral G protein-coupled receptors (vGPCRs), which are structurally and functionally similar to host GPCRs. Viral GPCRs play a crucial role in immunological processes within the cell. They were probably acquired from the host by molecular piracy and are now exploited by the virus to manipulate and evade the host's immune response. Thus, viral GPCRs are considered important drug targets for various diseases, therefore research of the pharmacological properties of these receptors and their mechanisms of endocytosis is a crucial step in their identifications as drug targets (10).

EBV encodes a viral G-protein-coupled receptor (vGPCR) called EBV-BILF1, which plays a crucial role in oncogenesis and immune evasion. EBV-BILF1 orthologs in pigs are encoded by three porcine lymphotropic herpesviruses (PLHV1—3) (11). The recent unveiling of the EBV-BILF1 structure by an international team, including Slovenian scientists, who described the specific features and highly conserved constitutive activity for Gαi coupling in a ligand-independent fashion, promises a significant advance in the formulation of strategies targeting the BILF1 receptors (3).

Endocytosis plays a crucial role in cellular transport of GPCRs. Several herpesviruses, including EBV, encode vGPCRs that help to evade the immune system and virus to spread. One of the published articles focused on the endocytosis of vGPCRs and their importance, highlighting the constitutive internalization of BILF1 from human and porcine γ-1 herpesviruses. New methods, such as real-time fluorescence assays, have played an important role in the study of these processes (1,2,4).

Furthermore, mechanisms of the internalization of the EBV-BILF1 receptor and receptor orthologs from porcine lymphotropic herpesviruses (PLHVs) were studied in detail in comparison to EBV-BILF1. A real-time fluorescence resonance energy transfer (FRET) assay together with the expression of dominant-negative variants of specific endocytic proteins, including dynamin-1 and the clathrin inhibitor Pitstop2 was used to investigate the mechanism of EBV-BILF1 internalization. Bioluminescence resonance energy transfer (BRET) saturation analysis was used to determine the interactions of the BILF1 receptors with β-arrestin2 and Rab7. Finally, the informational spectrum method (ISM) was used for bioinformatics analysis to investigate the interaction affinity of BILF1 receptors with various cellular components. Overall, this work showed that all BILF1 receptors undergo dynamin-dependent, clathrin-mediated constitutive endocytosis, that also dependent on caveolin-1 which is critical for proper BILF1 receptor trafficking. After receptor internalization recycling and degradation pathways have been proposed for BILF1 receptors. The study provides new insights into receptor transport and the similarities between the internalization mechanisms

Da bi dosegli tako resne komplikacije, so številni herpesvirusi, vključno z EBV, humanim citomegalovirusom (HCMV) in virusom, povezanim s Kaposijevim sarkomom (KSHV), razvili različne strategije za okužbo in vztrajanje v gostitelju vse življenje (8, 9). Ena od strategij vključuje izražanje virusnih s proteini G sklopljenih receptorjev (vGPCR-jev), ki so strukturno in funkcionalno podobni GPCR-jem gostitelja. vGPCR-ji igrajo ključno vlogo predvsem pri imunskih procesih v celici. Verjetno so jih od gostitelja pridobili s pomočjo molekularnega piratstva, sedaj pa jih virus izkorišča za manipulacijo in izogibanje imunskemu odzivu gostitelja. Tako vGPCR-ji veljajo za pomembne tarče zdravil za različne bolezni, zato je raziskava farmakoloških lastnosti teh receptorjev in njihovih mehanizmov endocitoze pomemben korak pri njihovi identifikaciji kot tarč zdravil (10).

EBV kodira z G-proteini sklopljen receptor (vGPCR), imenovan EBV-BILF1, ki je ključen pri onkogenezi in izmikanju imunskemu sistemu človeka. Ortologe receptorja EBV-BILF1 pri prašičih kodirajo trije gamaherpesni prašičji virusi (PLHV1—3) (11). Nedavno odkritje strukture receptorja EBV-BILF1, pri katerem so bili vključeni tudi slovenski raziskovalci, ki opisuje njegove strukturne značilnosti in visoko ohranjeno konstitutivno aktivnost za vezavo z Gαi podenoto proteina G v odsotnosti liganda, bo znatno pripomoglo k napredku pri raziskovanju receptorjev BILF1 kot obetajočih tarč za nova zdravila (3).

Endocitoza je pomembno vpletena v znotrajcelični transport, tudi GPCR-jev. Različni herpesvirusi, vključno z EBV, ki kodirajo vGPCR-je, pomagajo pri izogibanju imunskemu sistemu in širjenju virusa. Objavljeni pregledni članek obravnava endocitozo vGPCR-jev in njen pomen, s poudarkom na konstitutivni internalizaciji človeških in prašičjih γ-1 herpesvirusnih receptorjev BILF1. Nove metodologije, kot so metoda internalizacije v realnem času, ki temelji na metodi FRET, so bile ključne pri proučevanju teh procesov (1, 2, 4).

V enem izmed člankov, ki so jih objavili slovenski raziskovalci v sodelovanju s partnerji v tujini, so bili podrobno proučevani mehanizmi za internalizacijo receptorja EBV-BILF1 in translacijski potencial ortologov receptorja EBV-BILF1, ki jih kodirajo prašičji limfotropni herpesvirusi (PLHV) v primerjavi z EBV-BILF1. Za raziskovanje mehanizma internalizacije receptorjev BILF1 je bila uporabljena nova metoda, temelječa na fluorescenčnem prenosu resonančne energije v realnem času (FRET) skupaj z izražanjem dominantno negativnih mutant specifičnih proteinov, vključenih v proces endocitoze, vključno z dinaminom-1 in zaviralcem klatrina Pitstop2. Saturacijska analiza bioluminiscenčnega prenosa resonančne energije (BRET) je bila uporabljena za preučevanje interakcij receptorjev BILF1 z β-arestinom2 in Rab7. Poleg tega je bila za bioinformatično analizo uporabljena metoda informacijskega spektra (ISM) z namenom raziskovanja interakcijske afinitete receptorjev BILF1 z različnimi celičnimi proteini ali pododdelki. Študija je pokazala, da je endocitoza vseh receptorjev BILF1 konstitutivna in odvisna od dinamina

of EBV-BILF1 and PLHV1—2 BILF1 suggest potential translational applications for PLHVs (1).

Another study focused on the constitutive activity of EBV-BILF1 and its role in EBV-mediated immunosuppression and oncogenesis. The cryo-electron microscopy structure, resolved at 3.2 Å, showed that an extracellular loop within EBV-BILF1 obstructed the usual chemokine binding site, indicating EBV-BILF1 receptor activation without ligand, suggesting that the intrinsic activity of EBV-BILF1 underlies immunosuppression and virulence without being dependent on the presence of a ligand. This finding has implications in discovering novel functions of GPCRs encoded by similar viruses and for the development of antiviral therapies (3).

EBV-BILF1 has also been shown to play a critical role in oncogenesis and immune evasion by downregulating major histocompatibility complex (MHC-I) molecules in infected cells. This downregulation is thought to occur through the internalization of EBV-BILF1 together with MHC-I. In immunosuppressed transplant patients, EBV infection can lead to PTLD. Miniature pigs infected with PLHV1—3 develop a similar disease, which makes them potential preclinical model for PTLD. BILF1 orthologs encoded by PLHVs have similar characteristics to EBV-BILF1, including cell surface localization, internalization, MHC-I downregulation, and Gαi signaling patterns. PLHV1 was observed in the lymphoid tissues of pigs suffering from PTLD, indicating its involvement in PTLD infection. The lack of preclinical models to validate BILF1 receptors and study EBV-related diseases is currently a challenge. Nevertheless, the results of these recently published articles suggest that PLHV1-infected pigs may represent a viable model for studying the potential role of BILF1 as a key driver and therapeutic target in EBV-associated proliferative diseases (2).

As different efforts are made to find a new therapeutic possibility against EBV-related diseases recent paper in Science (12) pronounced the importance of finding new therapeutic possibilities against EBV-related diseases. In this paper latent gene EBNA-2, which initiates the transcription of viral and cellular genes and induces B-cell transformation and was exploited many years in connection with vGPCRs (13) was being shown to be involved in expression of metabolic enzyme IDO1 in infected cells. IDO1 controls immune responses and controls *de novo* synthesis of NAD+. Blocking IDO1 could therefore for be used as the first precision medicine cellular-metabolic intervention affecting viral infection *in vivo*.

This research is not only pushing the boundaries of what is known about EBV and developing this vital research area, but it is also attracting international acclaim. This work is attracting prestigious and ongoing collaborators from Denmark, Germany, Serbia, the United Kingdom and the United States, such as Stanford and Colorado Universities. The featured studies provided a comprehensive understanding of the role of EBV-BILF1 in viral pathogenesis and immune evasion as

ter poteka preko klatrinsko odvisne poti. Afiniteta interakcije med receptorji BILF1 in kaveolinom-1, skupaj z zmanjšano internalizacijo ob prisotnosti dominantno negativne variante kaveolina-1, je pokazala vpletenost kaveolina-1 v prerazporejanje receptorjev BILF1. Po internalizaciji so bile pri receptorjih BILF1 proučevane poti njihovega recikliranja in razgradnje. Študija odstira nov vpogled v znotrajcelično prerazporejanje receptorjev, podobnosti mehanizmov internalizacije med EBV-BILF1 in PLHV1—2 BILF1 pa kažejo na morebitne translacijske aplikacije za PLHV (1).

Naslednja študija se je osredotočala na konstitutivno aktivnost receptorja EBV-BILF1 in njegovo vlogo pri zaviranju delovanja imunskega sistema in pri virusni onkogenezi EBV. Struktura, pridobljena z uporabo krioelektronske mikroskopije pri ločljivosti 3,2 Å, je pokazala, da zunajcelična zanka receptorja EBV-BILF1 ovira običajno vezno mesto za kemokine, kar kaže na aktivacijo receptorja v odsotnosti liganda in pomeni, da aktivnost receptorja EBV-BILF1 vpliva na zaviranje imunskega sistema gostitelja in virulenco virusa, brez vezave liganda na receptor. Ta ugotovitev ima pomembne posledice za delovanje GPCR-jev, kodiranih s podobnimi virusi, in za razvoj terapij proti EBV (3).

Kot je bilo omenjeno, je receptor EBV-BILF1 ključen pri onkogenezi in izmikanju imunskemu sistemu, kar doseže z znižanjem površinske izraženosti glavnih molekul histokompatibilnega kompleksa (MHC-I) na okuženih celicah. Do znižane površinske izraženosti naj bi prišlo zaradi internalizacije receptorja EBV-BILF1 skupaj z molekulami MHC-I. Pri bolnikih z zavrtim imunskim sistemom po presaditvi tkiv lahko okužba z EBV povzroči posttransplantacijsko limfoproliferativno bolezen (PTLD). Miniaturni prašiči, okuženi s prašičjim limfotropnim herpesvirusom (PLHV1—3), razvijejo podobno bolezen, zaradi česar so potencialno zanimivi kot predklinični modeli za PTLD. Ortologi BILF1, kodirani na virusih PLHV, kažejo podobne lastnosti kot EBV-BILF1, vključno z lokalizacijo na celični površini, internalizacijo, znižano regulacijo molekul MHC-I in znotrajceličnim prenosom preko Gαi. PLHV1 so izsledili v limfatičnem tkivu prašičev, obolelih s PTLD, kar kaže na njegovo vpletenost v okužbo s to boleznijo. Kljub temu pomanjkanje predkliničnih modelov za validacijo receptorjev BILF1 in preučevanje bolezni, povezanih z EBV, predstavlja trenutni izziv. Vendar pa ugotovitve v nedavno objavljenih člankih razkrivajo, da bi lahko bili prašiči, okuženi s PLHV1, obetajoč model za raziskovanje potencialne vloge EBV-BILF1 kot ključnega igralca in terapevtske tarče pri proliferativnih motnjah, povezanih z EBV (2).

Veliko raziskovalnih skupin si prizadeva poiskati nove terapevtske možnosti proti boleznim, povezanim z EBV. Eden izmed prebojnih je nedavni članek, objavljen v Science (12), ki je poudaril pomen iskanja novih terapevtskih možnosti proti boleznim, ki so povezane z EBV. V tem prispevku je bilo dokazano, da je latentni gen EBNA-2, ki sproži transkripcijo virusnih in celičnih genov ter transformacijo B-celic in so ga mnogo let proučevali v povezavi z vGPCR (13), vključen v

well as novel insights into potential therapeutic strategies targeting this receptor.

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izražanje presnovnega encima IDO1 pri okuženih celicah. IDO1 nadzoruje imunske odzive in *de novo* sintezo NAD+. Blokiranje IDO1 bi se torej lahko uporabilo kot prva usmerjena medicinska celično-presnovna intervencija, ki bi vplivala na virusno okužbo *in vivo*.

Opisane raziskave premikajo meje znanega o EBV in razvijajo to ključno raziskovalno področje, hkrati pa so tudi mednarodno odzivne in omogočajo sodelovanje s prestižnimi sodelavci iz Danske, Nemčije, Srbije, Združenega kraljestva in Združenih držav, kot sta Univerza Stanford in Univerza v Koloradu. Nominirane in nagrajene študije omogočajo celovitejše razumevanje vloge receptorja EBV-BILF1 pri virusni patogenezi in izogibanju imunskemu sistemu ter vpogled v možne terapevtske strategije, ki ciljajo ta receptor.

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Review Article

Skin Dysbiosis in Atopic Dogs: Is Phage Therapy an Alternative to Antibiotics?

Key words

dysbiosis;
pyoderma;
canine atopic dermatitis;
bacteriophages;
phage therapy

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Abstract: Bacterial overgrowth, also known as dysbiosis, is a common concomitant of canine atopic dermatitis. Microbial diversity is decreased and coagulase-positive staphylococci are more abundant in dogs with canine atopic dermatitis compared to healthy dogs. Antimicrobial therapy restores the diversity of the skin microbiome; however, this effect can diminish after treatment is discontinued. Therapies for skin dysbiosis have traditionally included antibiotics and antiseptic medications. Due to increasing microbial resistance to antibiotics, the era of novel antimicrobial agents for the treatment of skin infections has already begun. Recent research highlights potential new treatment options, of which one of the most promising appears to be the use of bacteriophages. Bacteriophages are viruses that can infect and kill bacteria without having negative effects on human or animal cells. This article provides an update on human and veterinary research on phage therapy as a potential approach for the treatment of bacterial infections, with a focus on the treatment of skin dysbiosis in atopic dogs. The clear clinical potential of phage therapy, its advantages and disadvantages, and the legal, biological, technical, and economic challenges it faces for its further implementation and wider application are outlined.

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Introduction

Many skin diseases in humans and animals are associated with an imbalance in the skin microbiome, recently termed dysbiosis. The subtle stability of the skin's commensal community maintains the healthy state of the skin as it affects immune system functions and can rapidly change in response to environmental changes (1). The term dysbiosis describes "an altered composition of the commensal microbiome that is detrimental to the host" (2).

Canine atopic dermatitis (cAD) is similar to human atopic dermatitis, sharing clinical signs, altered epidermal barrier function, immune system dysregulation, and microbiome dysbiosis (3-11). Atopic dermatitis is the most common chronic inflammatory skin disease in humans and dogs, affecting around 20% of children, 2–7% of adults, and 10–15% of dogs worldwide, with local prevalences varying by region (12, 13). The diagnosis of atopic dermatitis is primarily a clinical diagnosis, based on clinical signs (on the face, intertriginous regions (e.g., axillae and groin), feet, and flexor surface of joints) and the exclusion of differential diagnoses (14, 15). The updated definition of cAD describes this disease in more detail as follows: a hereditary, typically pruritic and predominantly T-cell-driven inflammatory skin disease involving interplay between skin barrier abnormalities, allergen sensitization, and microbial dysbiosis (16).

Bacterial overgrowth (i.e., dysbiosis) and bacterial skin infection (i.e., pyoderma) are secondary in atopic dogs (see figure 1) (17, 18). It is not yet entirely clear whether dysbiosis is a trigger for or consequence of atopic dermatitis, or perhaps both (19). In human atopic dermatitis, *Staphylococcus aureus* has been shown to promote lesion formation (20, 21), and toxins produced by *S. aureus* are thought to trigger or exacerbate inflammation in atopic dermatitis (22). One such toxin is delta toxin, which has recently been shown to trigger mast cell degranulation and promote inflammatory skin disease (23).

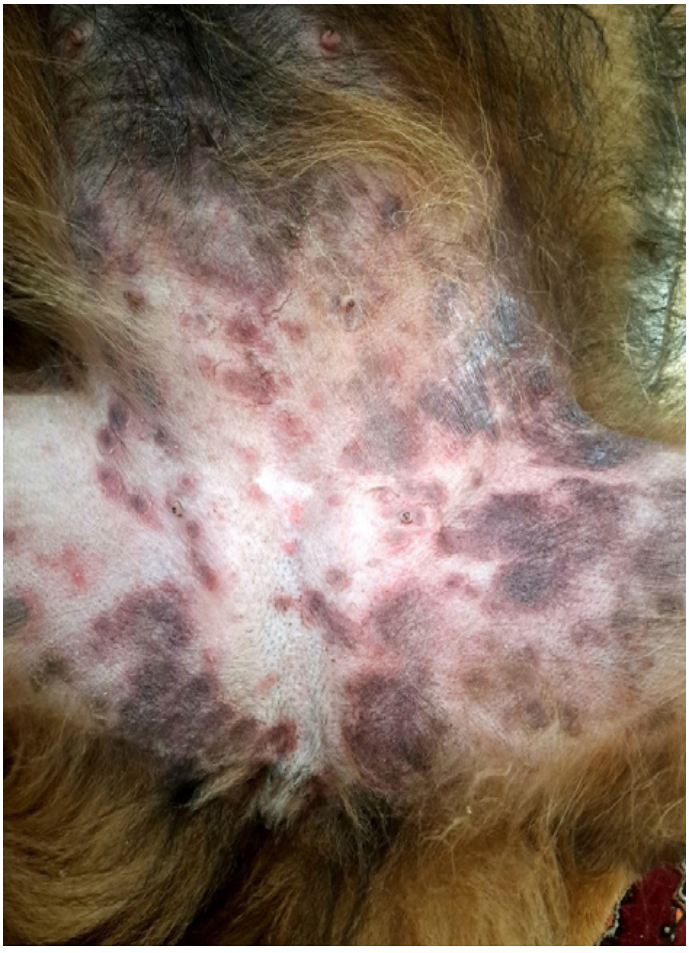

Figure1: Skin dysbiosis in a dog before treatment

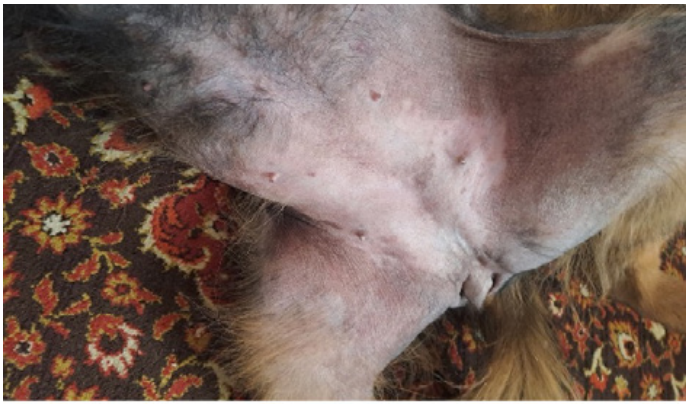

Figure 2: Clinical improvement in the same dog after treatment

The skin and nasal mucosa of humans (24-28) and dogs (9, 29-31) with atopic dermatitis are more frequently colonized with *S. aureus* and *Staphylococcus pseudintermedius*, respectively, compared with healthy patients. Veterinary studies demonstrate a significant decrease in microbial diversity and a higher abundance of coagulase-positive staphylococci in dogs with cAD (even on their apparently healthy skin) compared to healthy dogs (8, 9, 32, 33). Antimicrobial therapy can restore skin microbiome diversity (see figure 2 in comparison to figure 1, which displays dysbiosis in the same dog before treatment with antiseptic shampoo) (9, 24, 28, 34); however, the effect may diminish after treatment is discontinued (9).

Moreover, microbial resistance to antibiotics is increasing, and thus the era of novel antimicrobial agents for treating skin infections has already arrived. In line with the One Health approach, efforts should be made to efficiently manipulate the skin microbiome without the use of antibiotics, as this would significantly contribute to the prevention of bacterial resistance (35).

New options for skin dysbiosis treatment

Recent research indicates possible new treatment options (Table 1). Among the most studied new therapies is the use of bacteriophages (i.e., phage therapy). Bacteriophages are viruses that can infect and kill bacteria without negative effects on human or animal cells (72, 73). Their narrow spectrum of action avoids the main problems associated with antibiotics, such as affecting the entire microbiome by eliminating potentially beneficial bacteria, overgrowth of secondary pathogens, and the emergence of resistant bacteria (72). In addition, their ability to replicate only in target bacteria and their inability to infect mammalian cells makes their use much safer (74). The use of bacteriophages could also be more cost-effective than the use of antibiotics targeting multidrug-resistant pathogens (75). This article provides an update on human and veterinary research on phage therapy as a potential approach to the treatment of skin dysbiosis, particularly in cAD.

Phage therapy

Bacteriophages are the most common biological entity (76, 77). Similar to their bacterial hosts, bacteriophages are cosmopolitan, and an estimated 10^7 bacteriophage particles can be present in 1 mL of natural sample (78). Bacteriophages are found ubiquitously, anywhere bacteria survive, i.e., on marine and terrestrial surfaces and in soil, water, sewage, extreme environments, hospitals, and animal and human tissues (76). Several thousand bacteriophages have been described and classified according to their morphological characteristics, nucleic acid content, habitat, target bacterial species (75), and biological cycle (79). Classification based on biological cycle is the most useful, as it distinguishes between lytic (i.e., virulent) and lysogenic (i.e., temperate) bacteriophages and thus highlights differences regarding attachment to and invasion of bacteria (80). Lytic bacteriophages are of interest for the treatment of bacterial infections in humans and animals.

Bacteriophage activity is characterized by absolute specificity (75). To initiate binding, bacteriophage structures must match strain-specific variants of bacterial receptors. Both bacteriophages and bacteria are subject to constant mutations, resulting in a limited number of binding combinations,

Table 1: Alternatives to antibiotic treatment, apart from phage therapy

Antibiotic alternative	Aim of the studies	Results	Reference numbers
Probiotics	To review the current state of knowledge about gut microbial communities, advances in probiotic therapies, and whether the composition of the gut microbiome influences the composition of the skin microbiome and the pathogenesis of skin diseases.	Probiotics can help strengthen barrier function, reduce sensitivity, and modulate the immune system of the skin, enabling skin homeostasis.	36-40
Quorum quenching	To review natural anti-biofilm mechanisms recently identified in pathogenic, commensal, and probiotic bacteria.	Bioactive molecules that inhibit growth, interrupt quorum sensing, and/or prevent bacterial adhesion can prevent skin infections.	41-50
Antimicrobial peptides	To test whether various peptides can be used as diagnostic markers and for the treatment of different skin diseases.	Peptides have potential as diagnostic markers and for the treatment of skin diseases; however, further research is needed.	51-59
Gut and skin microbiome transfer (bacteriotherapy)	To investigate whether various skin diseases, such as atopic dermatitis, can be influenced by transmitted bacteria.	Transplantation of feces can suppress atopic dermatitis symptoms. Some bacterial strains can suppress *Staphylococcus aureus* in atopic dermatitis and improve inflammation.	10, 60-71

Table 2: The advantages and disadvantages of phage therapy

Advantages	Disadvantages
The ability to infect and kill bacteria without having negative effects on human or animal cells (77).	Preparation for clinical use is difficult (75, 113).
Significantly more effective than antibiotics owing to a very specific mechanism of action (75).	Bacteriophages might transfer antibiotic-resistant genes (75).
The occurrence of resistant bacteria is less likely (77).	The emergence of bacterial resistance to bacteriophages is possible (75, 123).
The entire microbiome is not affected, and potentially beneficial bacteria are not eliminated (77).	The activity of bacteriophages may be reduced by the response of the mammalian immune system to bacteriophages, and the specific bacteriophage activity for a particular bacterial strain may be absent regardless of the response of the mammalian immune system (75).
Only very few doses are needed (81, 82, 111).	
The effects are limited to accessible infection sites (83).	
Further advantages can be achieved with genetic engineering (93).	
May be less costly than antibiotic treatment (75).	

Legend: The numbers in brackets stand for the respective references

such that it is possible that a single bacteriophage binds to only a single bacterial strain (80). By contrast, in theory, no bacterium exists that cannot be lysed by at least one bacteriophage. Indeed, bacteriophages are much more effective than antibiotics due to their high specificity of action, which is their most attractive property (75). Unlike antibiotics, bacteriophages do not need to be administered in short succession over several days, as they can remain and multiply in the human or animal body for the duration of the infection (81, 82). As such, very few doses are required because the concentration of bacteriophages at the site of infection increases after the first administration (82). Unlike antibiotics, their effects are limited to the accessible site of infection (83).

Bacteriophages only kill the pathogen they can recognize, whereas antibiotics mostly have a very broad spectrum of action (75). Nevertheless, the idea of using bacteriophages in combination with antibiotics to treat bacterial infections has emerged (77). However, this can lead to antagonism because antibiotics often interfere with bacterial processes that are required for successful bacteriophage infection.

Additionally, antibiotics reduce the number of bacteria and thus decrease the ability of bacteriophages to proliferate (84, 85). By contrast, simultaneous treatment with bacteriophages and antibiotics at low (subinhibitory) concentrations can lead to so-called phage-antibiotic synergy (84-89). In an interesting study, a lytic bacteriophage was selected for *Pseudomonas aeruginosa* that uses an outer membrane porin that is part of a multidrug efflux system as a receptor, pressuring the host to mutate toward increased drug sensitivity to escape the bacteriophage (90). This is an approach that aims to resensitize multidrug-resistant pathogens to conventional antibiotics. Selected bacteriophages can be administered together with the antibiotic(s) to which they increase bacterial susceptibility (90-92). The advantages and disadvantages of phage therapy are summarized in Table 2, which clearly demonstrates the benefits of phage therapy.

Genetic engineering of bacteriophages

Genetic engineering can increase the therapeutic potential of bacteriophages (93). This can be directly achieved by modifying the host range (e.g., by homologous recombination or mutagenesis of tail fiber genes), bacteriophage infection (e.g., by deleting or deactivating genes required for lysogenic cycles), or the bacteriophage capsid (e.g., by selecting bacteriophages that can remain in the bloodstream longer). Bacteriophages can also be modified to enhance the antibacterial effects of conventional antibiotics, e.g., by enabling the production of factors that interfere with quorum sensing or enzymes that degrade biofilm matrices (84). For example, Lu and Collins modified a bacteriophage to express a biofilm-degrading enzyme that is effective against biofilm-producing *Escherichia coli* (94). Furthermore, it is possible to develop bacteriophages that combat bacterial resistance to antibiotics (75).

Bacteriophage-derived enzymes (enzybiotics)

Another therapeutic possibility is the use of bacteriophage-derived enzymes called enzybiotics. Currently, the greatest advances have been made with bacteriophage-encoded peptidoglycan hydrolases, which are highly effective against many clinically relevant pathogens. Interestingly, peptidoglycan hydrolases generally have broader specificity compared to whole bacteriophages (95, 96). Formulation options for enzybiotics range from liquids to dry powders, all of which can be stored for extended periods of time. Bacteriophage enzymes also tend to remain stable over wide pH ranges as well as at 4 °C and −80 °C (97). Junjappa et al. tested enzybiotic P128 hydrogel in 17 dogs with staphylococcal pyoderma. Daily treatment for 8 days resulted in complete recovery with no recurrence of symptoms for 2 months (96). Jun et al. tested the safety of the peptidoglycan hydrolase endolysin SAL-1 administered intravenously with increasing dosages once weekly in four dogs. Authors noted adverse side effects in 18.7% of administrations (3/16) when higher dosages were administered. Adverse events included subdued behavior, prone position, irregular breathing, vomiting, and transient changes in cardiovascular function (98). Overall, more comprehensive studies on phage therapy are needed to determine the safety and efficacy of enzybiotics.

The history of phage therapy

The first reports on bacteriophages were published in 1898, and a clear interest in using bacteriophages to treat bacterial infections in humans emerged after the researchers Twort and d'Herelle published their work in 1915 and 1917, respectively. In 1919, d'Herelle successfully used bacteriophage preparations to treat children suffering from bacterial dysentery, and phage therapy was widely used to treat bacterial infections in humans and animals in the 1930s, long before penicillin became available. Another study on phage therapy in humans was conducted and published as early as 1921 by the physician Bruynoghe and others (99).

The first program for phage therapy for human diseases was opened in what is now Tbilisi, Georgia, followed by another in Wroclaw, Poland; both programs still exist today. The G. Eliava Institute of Bacteriophages, Microbiology, and Virology in Tbilisi still houses a collection of bacteriophages isolated from environmental sources and collected in a bacteriophage bank. The collection provides a large repertoire from which bacteriophages can be either incorporated into preformulated products or selectively matched against bacterial isolates for personalized therapies (100). However, after World War II brought penicillin to the market in the early 1940s, phage therapy stopped in the West. The broad-spectrum activity of penicillin and later antibiotics against bacterial infections was considered an advantage over bacteriophages that require bacteria to express specific surface molecules to which the phage can bind. In addition, bacteria have intracellular defense mechanisms that can inactivate bacteriophages after invasion (101). The Cold War between the Eastern and Western blocs after World War II had a detrimental effect on scientific exchange between European countries and contributed to phage therapy being considered useless.

The new age of phage therapy research

Following the introduction of the last new family of antibiotics in 1987 and the emergence of resistant bacteria, researchers have once again started to focus on phage therapy. The number of clinical trials on the therapeutic use of bacteriophages is steadily increasing (101). Recent studies on human phage therapy have covered life-threatening diseases such as *P. aeruginosa* septicemia after liver transplantation (102), *P. aeruginosa* pulmonary infections in

cystic fibrosis (103), osteomyelitis in diabetic patients (104), infective endocarditis (73), and nontuberculous mycobacterium infections (105). Furthermore, reviews (100, 106) have covered more than 120 studies involving around 4000 human patients between 2000 and 2023 (107). These studies mostly reported cases of compassionate treatment. However, one prospective clinical trial involved patients with urinary tract infections treated with an adapted commercial bacteriophage drug provided by the George Eliava Institute of Bacteriophage, Microbiology and Virology, Tbilisi, Georgia (108).

Phage therapy is suitable for compassionate use due to its long-standing historical use, apparent lack of side effects, and supportive evidence from published research. Increasing media coverage and scientific articles have raised public awareness of the potential of phage therapy. However, compassionate phage therapies remain limited to a small number of experimental treatment centers or are performed by individual physicians and researchers. By establishing guidelines and increasing the availability of bacteriophages, we could enable compassionate phage therapies for more people in need (100). It is encouraging that the European Medicines Agency published guidelines on the quality, safety and efficacy of veterinary medicinal products specifically designed for phage therapy in October 2023 (109).

Phage therapy of skin dysbiosis

The case study series by DeWit et al. provide interesting clinical results regarding phage therapy of human skin dysbiosis with Staphefekt, an endolysin with endopeptidase and putative amidase activity. Rescue treatment with Staphefekt resulted in significant clinical improvement, with clinically relevant decreases in *S. aureus* abundance but not complete eradication (110). One clinical study included 24 patients suffering from chronic otitis externa for 2–58 years owing to infection with multidrug-resistant P. *aeruginosa*. Patients were randomized into two groups (of 12 patients each) treated with either a single dose of the commercial six-bacteriophage cocktail (Biophage-PA) or placebo. Significant clinical improvements and decreased *Pseudomonas* counts from baseline were observed in the phage-treated but not the placebo group. The study demonstrated bacteriophage replication in the patients and did not report any adverse reactions or local or systemic toxicity (111).

Treatment of *P. aeruginosa*-infected ear canals of dogs with the same bacteriophage cocktail used in the clinical study by Wright et al. described above (Biophage-PA) decreased clinical scores by 30% and *P. aeruginosa* counts by 67% in just 48 h. The numbers of bacteriophages increased compared to the administered dose by a mean of 99.1-fold (range 2.8–433.3-fold). No treatment-related inflammation or other adverse events were observed during the trial period (82). Recently, Silva et al. prepared a gel containing lytic bacteriophages for *S. pseudintermedius* suitable for transdermal permeation in dogs (112). A promising paper by Slovenian researchers has reported 20 staphylococcal-specific bacteriophages isolated from wastewater by enrichment with *Staphylococcus epidermidis* or *S. aureus* (113), and tests with *S. pseudintermedius* are continuing. These and other veterinary phage therapy trials are summarized in Table 3.

Commercial preparations of bacteriophages

Bacteriophages against *P. aeruginosa*, *Staphylococcus*, *Salmonella* spp., and other bacteria are commercially available in the US and EU markets (123). In Europe, Lysando AG has developed Artilysins®—endolysin-based drugs with antibacterial properties against Gram-positive and Gram-negative pathogens (97). Two commercial bacteriophage products are currently available for the treatment of skin infections, one for use in humans and the other for use in animals. Staphage Lysate (SPL)® (Delmont Laboratories, Swarthmore, PA, USA) is currently the only product approved for use in *Streptococcus canis* skin infections in the US (123). A phage lysate against *S. aureus* infections is available on the EU market under the trade name Stafal® (124). This product has been approved by the Czech State Institute for Drug Control for the topical treatment of staphylococcal skin infections in humans (125).

Limitations and challenges of phage therapy

Phage therapy can be considered the third important intervention for the treatment of bacterial infections after vaccines and antibiotics (84, 126). Although phage therapy has clear clinical potential, it faces regulatory, biological, technical, and economic challenges for its further implementation and wider adoption (84, 91).

Regulatory challenges

In the US, bacteriophages and their products (lysins) are considered drugs and should thus undergo the same process as chemical drugs to obtain regulatory approval for commercial production and use. In the EU, bacteriophages are considered medicinal products, defined by the European Medicines Agency as "a substance or combination of substances intended to treat, prevent or diagnose a disease or to restore, correct or modify physiological functions by exerting a pharmacological, immunological or metabolic action" (126). However, both US and EU regulators agree, at a minimum, that therapeutic bacteriophages should be

Table 3: Clinical trials with phage therapy in veterinary medicine

Aim of the study	Results	Reference
Evaluation of bacteriophage treatment for chronic *Pseudomonas aeruginosa* otitis in dogs.	Topical administration of the bacteriophage cocktail in the ear resulted in lysis of *P. aeruginosa* without apparent toxicity and thus has potential to be a convenient and effective treatment for *P. aeruginosa* otitis in dogs.	82
Evaluation of the antibacterial effects of endolysin P128 on *Staphylococcus* isolates responsible for canine pyoderma.	The endolysin P128 proved to be an effective and practical drug for the treatment of staphylococcal pyoderma in dogs.	96
Evaluation of the lytic activity of the staphylococcal bacteriophage phiSA012 and its endolysin Lys-phiSA012 against antibiotic-resistant staphylococcal strains isolated from infected canine skin.	Lys-phiSA012 proved to be a potential therapeutic agent for various staphylococcal infections, including methicillin-resistant *Staphylococcus pseudintermedius* infections of canine skin.	114
Evaluation of the host range of phage isolates and their ability to lyse antibiotic-resistant *P. aeruginosa* isolated from canine diseases.	The isolated phages were able to lyse many *P. aeruginosa* strains (28/39), including strains with high resistance to fluoroquinolones (4/6).	115
Investigation of the feasibility of bacteriophage therapy to combat *Escherichia coli* urinary tract infections in dogs and cats.	Most uropathogenic *E. coli* were susceptible to lysis by naturally occurring bacteriophages.	116
Investigation of the antimicrobial efficacy of nebulized phage therapy in a porcine model of pneumonia caused by *P. aeruginosa*.	Administration of large amounts of active phages by nebulization during mechanical ventilation is feasible. Rapid control of in situ infection by inhaled bacteriophages was achieved.	117
Determination of the therapeutic efficacy of the PaVOA phage compared to a phage cocktail or the cephalosporin antibiotic ceftriaxone in a model of *P. aeruginosa* skin infection in New Zealand rabbits.	Wound healing studies showed that the phage cocktail resulted in a high healing rate and accelerated skin remodeling and was more effective than ceftriaxone. The phage PaVOA had the ability to kill bacteria quickly.	118
Evaluation of the use of phage therapy for the prevention and treatment of fracture-related infections in a clinically relevant rabbit model.	The study provided a proof of concept for the use of phage therapy in a clinically relevant model for fracture-related infections.	119
Isolation and evaluation of the efficacy of bacteriophages with specific lytic activity against *Staphylococcus aureus* strains with low cure rates (biofilm-producing, multidrug-resistant, and methicillin-resistant *S. aureus* strains) in bovine mastitis.	Two phages belonging to the *Podoviridae* family with specific lytic activity against *S. aureus* were isolated from dairy farm effluents. Strains were susceptible to *Staphylococcus* phage M8 as follows: multidrug-resistant (4/20; 20%), methicillin-resistant (4/13; 31%), and biofilm-producing *S. aureus* (1/10; 10%).	120
Evaluation of the current literature on bacteriophage treatment in poultry farming.	Current literature on the treatment of various infections in poultry farms with phages was collected.	121
Two previously isolated phages were used to study the therapeutic effects against *Pseudomonas plecoglossicida* fish infections.	The mortality of fish receiving PPpW-3, PPpW-4, PPpW-3/W-4, and control fish not receiving phages was 53%, 40%, 20%, and 93%, respectively. The daily mortality of fish decreased at a constant level.	122

Legend: MDR: Multidrug resistant; MRSA: Methycillin resistant *Staphylococcus aureus*; SA: *Staphylococcus aureus*

classified as biological therapies that require compliance with well-defined regulatory frameworks and manufacturing and production requirements.

Demonstrating the efficacy of phage therapies in controlled clinical trials, of which there are only a very limited number to date, has been crucial in accelerating the development of regulatory frameworks (84), at least for veterinary medicine (109). However, the lack of definitive guidelines and regulations has made bacteriophages less attractive to pharmaceutical companies and funding agencies, making it difficult to conduct large-scale clinical trials to demonstrate the efficacy, safety, and stability of bacteriophages and their products. Although countries such as Georgia, Russia, and Poland have practiced phage therapy since its discovery, since very recently, they had no regulatory guidelines. In Poland, phage therapy is considered an "experimental treatment" as defined by the 2011 Polish Journal of Laws, Article 1634 and Article 37 of the Declaration of Helsinki (127, 128). Veterinary bacteriophage production has recently been included in the European Medicines Agency guidelines, which specifically refer to bacteriophage products. However, bacteriophage-derived products (e.g., lysins or other enzymes) or magistral formulae consisting of bacteriophages are not within the scope of these guidelines (109).

Technical and biological challenges

The technical difficulty in the production of bacteriophage drugs is that the stability of the preparations for clinical use is strictly bacteriophage-dependent and that the

stabilization strategies must be optimized individually for each bacteriophage (129). This may lead to costly and time-consuming clinical trials, which discourage the pharmaceutical industry from researching and manufacturing bacteriophage preparations (75). Isolation of bacteriophages, usually from wastewater and feces, is the first step and is relatively straightforward (130). However, before identifying a bacteriophage as a potential therapeutic agent, its specificity to a particular bacterial strain must be demonstrated. This is quite challenging because detecting the lytic capacity of a bacteriophage depends on the interactions between the bacteriophage and bacterium and how they change over time along with the dose of bacteriophages used for the assay.

The bacteriophage genome must also be sequenced and cannot contain integrase genes (as in the lysogenic type), antibiotic resistance genes, genes for phage-encoded toxins, or genes for other bacterial virulence factors (131). In addition, bacteriophage activity may be reduced due to the immune system's response to bacteriophages, and specific bacteriophage activity for a given bacterial strain may be absent regardless of the immune system's response (75). There is also the possibility of bacterial resistance to bacteriophages evolving, as bacteria possess and can evolve different mechanisms to prevent viral infections (84, 132). The development of bacterial resistance to bacteriophages can be reduced by using bacteriophage cocktails, administering a higher initial bacteriophage inoculum, or combining bacteriophages with antibiotics. A higher inoculum is associated with a lower risk of developing bacteriophage-resistant bacteria because the bacteriophages kill pathogens faster than they can replicate (133).

Although the development and marketing of bacteriophage-based products is difficult under current regulations in both the US and EU, so-called "compassionate use of phage therapy" is permitted on a case-by-case basis, particularly for patients who have not responded to conventional therapies and are unable to participate in clinical trials. In the EU, phage therapy in humans has been successfully implemented at the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy in Wroclaw, Poland, and at the Queen Astrid Military Hospital in Brussels, Belgium (127).

Summary

For now, antibiotics will remain the standard clinical treatment for bacterial infections despite increasing antimicrobial resistance and multidrug-resistant infections. Nevertheless, in the near future, the search for new antimicrobial agents that act synergistically with antibiotics will be an important focus of drug development. It has already been demonstrated that subinhibitory concentrations of multiple antibiotic classes have a positive effect on bacteriophage plaque size and bacteriophage multiplication efficiency. However, a better understanding of the interactions between bacteriophages and antibiotics warrants further studies. Overall, combining bacteriophages with antibiotics can lead to synergies that should be exploited to improve antibiotic efficacy and add viable combination therapies to the clinical armamentarium.

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Disbioza kože pri atopičnih psih: ali je zdravljenje z bakteriofagi lahko alternativa zdravljenju z antibiotiki?

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Izvleček: Bakterijsko preraščanje, poimenovano tudi disbioza, pogosto spremlja atopijski dermatitis pri psih. Pri bolnih psih je v primerjavi z zdravimi opazna zmanjšana mikrobna raznovrstnost, prevladujejo pa koagulazno pozitivni stafilokoki. Protimikrobno zdravljenje sicer obnovi pestrost mikrobioma, vendar učinek lahko hitro mine, ko z zdravljenjem prenehamo. Disbiozo kože običajno zdravimo z antibiotiki in antiseptiki. Novi načini zdravljenja so zaradi naraščajoče odpornosti bakterij proti antibiotikom že našli svoje mesto v raziskavah. Med njimi se uporaba bakteriofagov zdi ena izmed bolj obetavnih potencialnih možnosti zdravljenja. Bakteriofagi so virusi, ki okužijo in ubijejo bakterije, ne da bi imeli negativen vpliv na živalske ali človeške celice. Članek povzema najnovejše raziskave v veterinarski in humani medicini s področja zdravljenja bakterijskih okužb z bakteriofagi. Še posebej se osredotoča na zdravljenje disbioze kože pri psih z atopijskim dermatitisom. V članku avtorici izpostavita jasen klinični potencial uporabe bakteriofagov pri zdravljenju, prednosti in slabosti tega zdravljenja ter pravne, biološke, tehnične in ekonomske izzive, s katerimi se raziskovalci soočajo v želji po uvedbi tega načina zdravljenja v širšo uporabo.

Ključne besede: disbioza; piodermija; pasji atopijski dermatitis; bakteriofagi; zdravljenje s fagi

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Original Research Article

The Effect of Ascending Doses of Ketoprofen on Biochemical and Coagulation Parameters in Lambs

Key words

drug safety;
haemostatic function;
hepatotoxicity;
ketoprofen;
lambs;
nephrotoxicity;
non-steroidal anti-inflammatory drug

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Abstract: Ketoprofen (KTP) is a non-steroidal anti-inflammatory drug (NSAID) used as an analgesic, antipyretic and anti-inflammatory agent in human and veterinary medicine. Although KTP is used in the treatment of diseases such as musculoskeletal inflammation, endotoxemia, pneumonia, enteritis in sheep and minor surgical procedures such as dehorning and castration there is no information about its safety. The aim of this study is to determine the effect of KTP on biochemical and coagulation parameters following intramuscular (IM) administration of different doses of KTP to lambs. In the study, 18 clinically healthy lambs were randomly divided into three groups of 6 animals each. KTP was administered IM to lambs at doses of 1.5, 3 and 6 mg/kg. Biochemical and coagulation parameters were evaluated by taking blood samples before drug administration (0 hour) and at 24 hours and 48 hours after administration. No local or systemic side effects were observed in lambs after the administration of KTP at different doses. The aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LDH) values at 24 hours significantly increased compared to 0 hours in all dosage groups ($p<0.05$). KTP did not cause a significant change in albumin (ALB), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (CRE), CK and LDH values in different dose groups. The AST value was only significantly higher in the 6 mg/kg dose group compared to the 1.5 mg/kg dose group at 24 hours ($p<0.05$). Although there was no statistically significant difference in intragroup prothrombin time (PT), fibrinogen and D-dimer levels in all dose groups, a significant increase was observed in the activated partial thromboplastin time (aPTT) value of 6 mg/kg dose group at the 24 hours compared to the 0 hour ($p< 0.05$). As a result, after IM administration of 1.5, 3 and 6 mg/kg, increased CK and LDH values, which may be associated with muscle damage, may limit use of KTP via IM injection in lambs.

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), which are widely used in painful and inflammatory conditions such as lameness, foot rot, castration, vasectomy, mastitis and laparoscopy in sheep, act by inhibiting the effect of the cyclooxygenase enzyme, which is responsible for the synthesis of prostaglandins (1-3). Ketoprofen (KTP), is a NSAID belonging to the arylpropionic acid group and exists as a chiral compound with R(-) and S(-) isomers present in the racemic formulation. In animals, the S(-) form is more active and there is interconversion between the forms (4). In sheep, the suppression of prostaglandin E2 (PGE2) and thromboxane B2 (TXB2) production has been reported for up to 12 hours following a single intravenous injection of 1.5 or 3 mg/kg of KTP (5). In addition, a study in sheep reported that the 4 mg/kg subcutaneous administration of KTP at 90 minutes before the procedure failed to alleviate the post-surgical pain response (6).

It is known that NSAID therapy has serious side effects such as gastrointestinal ulceration or bleeding, liver and

kidney damage, allergic reactions, myocardial infarction and cardiac sudden death. Coagulation, hematological and biochemical parameters are used to evaluate the effects of drugs on physiological and pathological conditions (7,8). Coagulation parameters (aPTT, PT, fibrinojen, D-dimer) reflect coagulation disorders, while hematological parameters (WBC, RBC, hemogram, hematocrit, platelet) reflect bone marrow functions and fluid electrolyte balance, biochemical parameters (albumin, ALP, ALT, AST, BUN, cholesterol, CK, creatinine, GGT, TP and triglyceride) reflect liver, kidney, muscle and lipid metabolism functions (9,10).

In studies conducted in calves, horses, dogs, pig and children, it has been shown that the administration of KTP at recommended doses does not cause any side effects (11-15). On the other hand, some studies in laboratory animals have reported that KTP administered at recommended therapeutic doses causes side effects such as gastrointestinal irritation, impaired kidney function, hepatopathies, prolonged bleeding and clotting times (13,16). In some cases, NSAIDs may need to be used in high doses. However, high doses of this group of drugs may cause undesirable effects. In the literature review, no reference was found regarding the effect of increasing doses of KTP on biochemical and coagulation parameters in lambs. The aim of this study was to determine the effects of KTP on biochemical and coagulation parameters after single doses of 1.5, 3 and 6 mg/kg in lambs.

Material and Methods

Animals

The study was performed on a commercial farm located in the district of Kadınhanı, Konya Province, Türkiye. The study was performed on 18 female Akkaraman lambs (3-6 months old, 23-36 kg) who were determined to be healthy in the general clinical examination and had not received any medication treatment in the one months prior to the study. The animals were divided into different groups 7 days before the start of the study and numbered with oily paint. The lambs were fed with commercial feed (CP-5621, Ankara, Türkiye) twice a day and alfalfa hay, grass hay and water were given ad libitum. All procedures on animals were approved of the Local Ethics Committee for Animal Experiments at Selcuk University on December 27, 2022 with the approval number 2022/140.

Experimental design

A total of 18 lambs were randomly divided into three groups of 6 animals each. KTP (100 mg/ml, Ba-Keto, Injection Solution, Bavet, Turkey) was administered as a single dose via intramuscular injection into the neck region of the lambs in the first, second and third groups at doses of 1.5 mg/kg, 3 mg/kg and 6 mg/kg, respectively, following the dosage references from previous studies (5,6). In the study, blood samples were collected from the jugular vein at 0, 24 and 48 hours using a venipuncture technique. Blood samples were collected into gel tubes (2 ml) for biochemical analysis and into sodium citrate tubes (2 ml) for coagulation tests. After centrifuging the blood samples at 3,500g for 10 minutes, the resulting serum and plasma samples were carefully transferred into 2 ml Eppendorf tubes. The serum samples were stored in a deep freezer at -80°C until the day of analysis, while the plasma samples were analyzed within 3 hours for coagulation tests. Throughout the experimental protocol, the injection site was monitored for swelling, redness and pain and the animals were closely observed for any clinical changes.

Analysis of biochemical parameters

Biochemical parameters including serum albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatine kinase (CK), creatinine (CRE) and lactate dehydrogenase (LDH) levels were determined using an automated analyzer device (Lab-300plus, Instrumentation Laboratory, Milano, Italy) from serum samples stored at -80°C.

Analysis of coagulation parameters

In plasma samples, the measurement of clotting factors including prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen (FIB) and D-Dimer was performed using a coagulation analysis device (Siemens, A-7799, Sysmex CA 1500, Germany).

Statistical analysis

The research data was presented as mean ± standard deviation (SD). Statistical analysis was conducted using the SPSS software (version 26.0, IBM). Biochemical and coagulation parameters were analyzed for homogeneity of variances. Values that showed normal distribution with p-values > 0.05 were considered for parametric statistical analysis. In both intragroup and intergroup comparisons of data, one-way analysis of variance (ANOVA) and post-hoc Tukey test were used (SPSS 26.0). $P < 0.05$ value was considered statistically significant.

Results

No adverse reactions, general (in their feeding, drinking, defecation and behavior) or local (pain, swelling, redness), were observed during clinical observation of lambs following intramuscular administration of KTP at doses of 1.5, 3 and 6 mg/kg.

The effect of KTP on the biochemical parameters of lambs after intramuscular administration at doses of 1.5, 3 and 6 mg/kg is presented in Table 1. In the evaluation within the group, there was no statistically significant difference in

Table 1: The effects on biochemical parameters of intramuscular administration of ketoprofen at doses of 1.5, 3 and 6 mg/kg to lambs (n=6, mean ± SD)

Parameters	Sampling Time (Hour)	1.5 mg/kg	3 mg/kg	6 mg/kg
ALB (g/L)	0	29.83±5.91	33.25±3.03	32.58±4.34
	24	34.25±1.72	35.33±3.33	34.42±2.73
	48	33.66±1.63	35.08±6.74	33.33±3.63
ALT (U/L)	0	16.08±3.64	19.17±3.60	17.92±4.76
	24	19.67±6.22	22.75±7.44	24.08±6.59
	48	18.33±4.89	15.50±5.00	19.33±1.08
AST (U/L)	0	97.25 ± 16.67^{y}	104.00 ± 12.15^{y}	97.17 ± 6.35^{z}
	24	$147.17\pm10.44^{b,x}$	$164.08\pm12.71^{ab,x}$	$180.33\pm13.85^{a,x}$
	48	115.42 ± 13.25^{y}	121.33 ± 29.44^{y}	124.58 ± 20.31^{y}
BUN (mg/dL)	0	72.08±6.71	73.33±9.01	73.75±6.05
	24	75.58±7.79	70.25±7.11	70.92±8.79
	48	70.58±6.33	71.58±5.39	70.25±7.24
CRE (mg/dL)	0	0.65±0.04	0.64±0.06	0.68±0.04
	24	0.79±0.18	0.76±0.16	0.71±0.13
	48	0.72±0.14	0.61±0.14	0.72±0.15
CK (U/L)	0	223.75 ± 22.94^{y}	243.58 ± 11.00^{y}	231.33 ± 41.68^{y}
	24	399.25 ± 73.72^{x}	384.42 ± 38.51^{x}	426.33 ± 145.25^{x}
	48	252.67 ± 35.07^{y}	268.25 ± 16.58^{y}	285.92 ± 55.26^{y}
LDH (U/L)	0	620.00 ± 47.18^{y}	640.92 ± 35.72^{y}	629.92 ± 47.10^{y}
	24	891.50 ± 128.45^{x}	880.25 ± 152.23^{x}	877.58 ± 153.09^{x}
	48	827.00 ± 81.93^{x}	830.50 ± 113.06^{x}	818.08 ± 140.62^{x}

^{a,b} Superscripts with different letter in the same raw show statistically significant differences ($P < 0.05$). ^{x,y,z} Superscripts with different letter in the same column show statistically significant differences ($P < 0.05$).

ALB; Albümin, AST; aspartate aminotransferase, ALT; alanine aminotransferase, BUN; blood urea nitrogen, CRE; Creatinine, CK; creatine kinase and LDH; lactate dehydrogenase.

ALB, ALT, CRE and BUN values in all dose groups ($p>0.05$). However, the AST, CK and LDH values at 1.5, 3 and 6 mg/kg doses increased at 24 hours compared to 0 hour ($P<0.05$). The AST and CK values at 1.5, 3 and 6 mg/kg doses decreased at 48 hours compared to 24 hours ($P<0.05$). There was no statistically significant difference in ALB, ALT, BUN, CRE, CK and LDH values between the groups ($P>0.05$). However, AST value in the 6 mg/kg dose group at 24 hours was significantly higher than that in the 1.5 mg/kg dose group ($P<0.05$).

The effect of KTP on coagulation parameters in lambs at doses of 1.5, 3 and 6 mg/kg after intramuscular administration is presented in Table 2. In the within-group evaluation, there was no statistically significant difference in PT and D-Dimer values in all dose groups ($P>0.05$). However, the aPTT value increased significantly at 6 mg/kg at 48 hours compared to 0 hours ($P<0.05$). In the evaluation between groups, there was no statistically significant difference in PT and D-Dimer values ($P>0.05$). However, the aPTT value in the 3 mg/kg dose group was significantly lower at 48 hours compared to the value in the 1.5 mg/kg dose group

Table 2: The effects on coagulation parameters of intramuscular administration of ketoprofen at doses of 1.5, 3 and 6 mg/kg to lambs (mean ± SD)

Parameters	Sampling Time (Hour)	1.5 mg/kg	3 mg/kg	6 mg/kg
PT (sn)	0	12.80±0.47	13.42±1.13	13.22±1.28
	24	14.65±2.32	13.55±1.15	12.87±1.03
	48	12.75±0.66	12.70±0.74	13.42±1.01
aPTT (sn)	0	38.17±0.83	36.47±2.46	37.28±2.04^{y}
	24	39.30±1.87	35.85±1.20	40.53±7.21^{xy}
	48	40.75±3.03^{a}	35.62±3.15^{b}	44.40±1.75^{a,x}
Fibrinogen (g/L)	0	2.21±0.17	1.88±0.25	1.96±0.28
	24	1.53±0.56^{b}	2.35±0.50^{a}	1.60±0.55^{ab}
	48	1.98±1.05	2.41±0.56	2.41±0.75
D-DIMER (ng/L)	0	457.67±64.97	501.83±85.35	535.83±51.57
	24	551.00±57.84	487.33±104.93	501.33±114.63
	48	524.67±82.46	518.50±62.91	512.00±63.22

^{a,b} Superscripts with different letter in the same raw show statistically significant differences (P < 0.05). ^{x,y} Superscripts with different letter in the same column show statistically significant differences (P < 0.05). PT; prothrombin time, aPTT; activated partial thromboplastin time

and the fibrinogen value in the 3 mg/kg dose group was significantly higher at 24 hours compared to the value in the 1.5 mg/kg dose group (P<0.05).

Discussion

KTP, a widely used NSAID in humans and animals, is a potent cyclooxygenase inhibitor. Unlike other NSAIDs, it also inhibits lipoxygenase and creates a double blockade in arachidonic acid metabolism. The recommended dose of KTP is 2 mg/kg for cats and dogs, 2.2 mg/kg for horses and 3 mg/kg for cattle and small ruminants (4,16). Since the effect of KTP varies depending on the dose, it can be applied in increasing doses (17,18), but undesirable effects may occur when used in increasing doses. The doses (1.5, 3 and 6 mg/kg) selected in this study and sample collection times were determined by considering previous studies in pigs, sheep and cattle (5,19,20). Common side effects of KTP are observed when it is used at higher doses than recommended or for a prolonged period of time. However, side effects are rarely reported after a single administration at the recommended dose (21). KTP administered intravenously at a dose of 3 mg/kg for 5 days was well tolerated in calves (11).

It has been found that gastrointestinal side effects tend to occur when dogs are given KTP for 30 days, but the lesions healed after administration have been discontinued (13). Horses did not show any clinical adverse effects during administration of 2.2 mg/kg three times daily KTP (12). Oral administration of KTP in children has been reported to be well tolerated for up to three weeks (15). In pigs, there was no change in serum biochemical values at the single intramuscular dose of 3, 6 and 9 mg/kg and 3 mg/kg for 3 days (14). In this study, no side effects were observed after IM administration of 1.5, 3 and 6 mg/kg KTP to lambs, but statistically significant intra and intergroup differences were observed in some biochemical and coagulation parameters. Most of the reports on the side effects of KTP administered at recommended treatment doses are obtained from humans and laboratory animals. These effects include gastrointestinal irritation, impaired kidney function, hepatopathies, prolonged bleeding and clotting times and photosensitivity (10,14). Gastrointestinal ulceration occurs due to inhibition of PGE2 synthesis and decreased production of mucosal protective agents (22). Nephrotoxicity is related to the inhibition of prostaglandins in the kidneys that are essential for salt and water balance, vascular tone, blood flow and renin secretion (23). Coagulation disorders are the result of thromboxane A2 deficiency in platelets after administration of COX1 inhibitors (24). Phototoxicity results from damage to cell membranes by radical intermediates (25).

In this study, AST, CK and LDH values increased at 24 hours compared to 0 hours in all dose groups (P<0.05). It was observed that AST and CK values decreased (P<0.05) in 48 hours compared to 24 hours, but LDH value did not change. The increase in serum CK and LDH levels may occur due to muscle damage caused by IM drug administration (26,27).

The halflife of CK is approximately 2 hours (28), thus indicating that the transient increase in serum CK level seen in this study is due to IM drug administration. In our study, it was concluded that mild and transient increases in enzyme levels, particularly within the same dosage groups, are related to the metabolism and elimination processes of KPT. In the current study, it was determined that KTP did not cause a significant change in ALB, ALT, BUN, CRE, CK and LDH values in different dose groups. Only the AST value in the 6 mg/kg dose group was significantly higher at the 24 hours compared to the 1.5 mg/kg dose group ($p<0.05$). It was determined that the elevated AST level returned to its normal levels in the 48 hours. AST also known as serum glutamate-oxaloacetate transaminase (SGOT), is found mainly in the heart, liver, kidneys and muscle tissue and high blood concentrations indicate liver damage or disease (29). Serum AST and ALT levels can be used as an indicator of liver damage. ALT is more liver specific than AST. AST instead of ALT can only be used when ALT is not present and there is no known muscle pathology causing an increase in AST (30). In previous studies, it was reported that KTP administration increased the AST enzyme in mice (31), dogs (32) and donkeys (33). These results are consistent with the results of our study. Low elevations in serum enzyme levels have been reported during KTP therapy and this has rarely been associated with significant acute liver injury (31).

KTP blocks the formation of thromboxane A2 by inhibiting thromboxane cyclooxygenase. It creates a systemic bleeding tendency by disrupting platelet aggregation and thus prolonging bleeding time (34, 35). In this study, although no statistically significant difference was found in intragroup PT, fibrinogen and D-dimer levels in all dose groups, a significant increase was observed in aPTT value at 6 mg/kg in 24 hours compared to 0 hour ($p<0.05$). PT and aPTT are commonly used coagulation measurement parameters in the evaluation of secondary hemostasis in humans and animals (36). aPTT level in healthy sheep has been reported to be between 29.2 ± 3.2 and 41.1 ± 8.7 seconds (37). It was reported that the administration of flunixin meglumine and meloxicam in sheep prolonged aPTT, but these changes were not statistically significant (38). It has been determined that carprofen increased the aPTT value in dogs on the 5, 7 and 12 days after administration (36). It has been reported that the administration of meloxicam and KTP in ponies did not cause any difference in fibrinogen, PLT, PT and aPTT values and did not affect the coagulation parameters (39). In this study, it was concluded that the fact that the fibrinogen value in the 3 mg/kg dose group was higher at 24 hours compared to the value in the 1.5 mg/kg dose group was not significant.

Conclusion

In conclusion, after IM administration of 1.5, 3 and 6 mg/kg of KTP, it was observed that CK and LDH values, which may be associated with muscle damage, were increased. This may limit use of KTP via IM route in lambs. In all dose groups, AST values, which may be associated with metabolism processes of KTP, were increased. The aPTT value increased at the dose of 6 mg/kg. For the effective and safe use of KTP in lambs, its hematological, biochemical, molecular and pathological safety must be demonstrated after repeated administrations and other administration routes.

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Vpliv naraščajočih odmerkov ketoprofena na biokemične in koagulacijske parametre pri jagnjetih

M. N. Ural, K. Üney

Izvleček: Ketoprofen (KTP) je nesteroidno protivnetno zdravilo (NSAID), ki se v humani in veterinarski medicini uporablja kot sredstvo proti bolečinam, povišani temperaturi in vnetju. Čeprav se KTP pri ovcah uporablja za zdravljenje mišično-skeletnih vnetij, endotoksemije, pljučnice, enteritisa in pri manjših kirurških posegih, kot sta odstranjevanje rogov in kastracija, ni podatkov o varnosti zdravila. Namen te študije je bil ugotoviti vpliv biokemijskih in koagulacijskih parametrov po intramuskularni (IM) aplikaciji različnih odmerkov KTP pri jagnjetih. Vrednosti aspartataminotransferaze (AST), kreatin kinaze (CK) in laktat dehidrogenaze (LDH) so se po 24 urah v primerjavi z 0 urami v vseh skupinah znatno povečale ($p < 0,05$). KTP ni povzročil značilnih sprememb vrednosti albumina (ALB), alanin aminotransferaze (ALT), dušika sečnine v krvi (BUN), kreatinina (CRE), CK in LDH v različnih skupinah odmerkov. Vrednost AST je bila po 24 urah pomembno višja le v skupini z odmerkom 6 mg/kg v primerjavi s skupino z odmerkom 1,5 mg/kg ($p < 0,05$). Čeprav znotraj posameznih skupin ni bilo statistično pomembnih razlik v vrednostih protrombinskega časa (PT), fibrinogena in D-dimerov, smo v skupini z odmerkom 6 mg/kg v 24 urah v primerjavi z uro 0 opazili znatno povečanje vrednosti aktiviranega delnega tromboplastinskega časa (aPTT) ($p < 0,05$). Posledično bi lahko povečane vrednosti CK in LDH (ki so lahko povezane s poškodbami mišic) omejile uporabo IM aplikacije KTP pri jagnjetih.

Ključne besede: varnost zdravil; hemostatska funkcija; hepatotoksičnost; ketoprofen; jagnjeta; nefrotoksičnost; nesteroidno protivnetno zdravilo

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Original Research Article

Evaluation of the Effect of Temperature on the Toxicity of Lambda-Cyhalothrin in *Dreissena Polymorpha* Using some Biochemical Biomarkers

Key words

λ-cyhalothrin;
D. polymorpha;
oxidative Stress;
neurotoxicity;
temperature

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Abstract: Due to increasing climate change, it has become important to determine whether the dose-response relationship of organisms to some substances is affected by temperature. For this reason, in this study, it was aimed to reveal the effect of the temperature variable on the toxic response using the *Dreissena polymorpha* model organism and some of its biomarkers. For this purpose, acetylcholinesterase (AChE), catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) levels in *Dreissena polymorpha* exposed to subletal concentrations of λ-cyhalothrin at different temperatures were measured using commercial ELISA kits. According to the results obtained, there was a statistically significant increase in MDA levels in the groups exposed to λ-cyhalothrin, while a decrease in GSH levels was found. AChE levels were inhibited especially in the groups exposed high concentrartion of λcyhalothrin. It was also found that the inhibition levels increased depending on the application times. While SOD enzyme activity decreased, CAT enzyme activity increased depending on the exposure concentration. It has been observed that different temperature have different effects on the toxicity of λ-cyhalothrin. It was observed that λ-cyhalothrin caused oxidative stress and neurotoxicity, and the toxicity of λ-cyhalothrin changed depending on the temperature.

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Introduction

Lambda-cyhalothrin (λ-cyhalothrin) is a synthetic pyrethroid insecticide. It is used in agriculture, household pest control, food preservation and disease vector control (1). There is limited information on the environmental concentrations in surface waters of these pyrethroids whose are widely used around the World (2, 3). λ-cyhalothrin concentrations in surface waters range from 346 ngL^{-1} (4) to 797 ngL^{-1} (5). It is known that pesticides cause serious toxicological effects and biochemical dysfunctions. Recent studies (6, 7) have shown that λ-cyhalothrin cause neurotoxicity, hepatotoxicity and oxidative damage.

λ-cyhalothrin itself does not directly generate free radicals, but indirectly contributes to the formation of various types of radicals such as superoxide radical, peroxynitrite, nitric oxide and nitrogen species such as hydroxyl radical, causing oxidative stress (8, 9). These radicals cause lipid peroxidation in the cell membrane, leading to destabilization and fragmentation of the membrane (10). Lipid peroxidation, induced by reactive oxygen species (ROS), is a common oxidative stress biomarker of toxicants. Lipid peroxidation caused by ROS is the most important biomarker of oxidative stress (11). The MDA, the most important indicator of lipid peroxidation, is a secondary product of lipid peroxidation (12). It is known that pyrethroids show their neurotoxicity mainly by interfering with the function of sodium channels and calcium-dependent chloride channels in the central nervous system (13). λcyhalothrin can cause neurotoxicity in non-target aquatic organisms and aquatic invertebrates through acetylcholine, a neurotransmitter substance (14,

15, 16). It has been shown in studies that changes in AChE activity are used as potent biomarkers for organophosphorus and carbamate pesticides (17).

GSH, which is a part of the secondary defense system, is a non-enzymatic antioxidant and plays a role in scavenging free radicals (18). Reduced GSH protects cell membranes from lipid peroxidation and non-protein thiol and is one of the main reducers found in cells (19).The most important defense mechanisms against the toxic effects of oxygen metabolism are SOD and CAT. SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, whereas CAT; converts hydrogen peroxide into water. These enzymes play a very important role in mitigating the toxic effects of ROS (20).

D. polymorpha, commonly known as the zebra mussel, is native to the Palearctic region of the world, the freshwater drainage basins of the Caspian and Black Sea, and the Dniester, Volga, Danube, and Ural rivers (21). Climate change is causing rivers and lakes to warm and glaciers to shrink, which in turn changes the quantity and quality of melt water (22). Because it is sensitive to environmental changes, one of the most valuable invertebrate models for freshwater ecotoxicological studies is the bivalve zebra mussel (*Dreissena polymorpha*), which has been extensively used for biomonitoring of organic pollutants and for the evaluation of several biomarkers, both in vitro and in vivo (23).

Temperature, one of the main environmental stressors, can interact with insecticides (24). It has been studied in many aquatic organisms, including amphibians, crustaceans, annelids and arthropods, where chemical pollutants may interact with natural stressors (eg, temperature, predation, larval competition) (25).

In this study, we evaluated the effects of λ-cyhalothrin on *D. polymorpha* at different temperatures to determine whether temperature and pesticides interact synergistically on some biochemical biomarkers in *D. polymorpha*.

Material and Methods

Chemicals

All chemicals were used directly without further purification. λ-cyhalothrin was purchased from local chemicals market from Turkey. The studied concentrations were prepared by diluting commercially purchased λ-cyhalothrin with distilled water.

Model organism

The model organism *D. polymorpha* individuals used in the study were obtained from culture collection of Fiseheries Labaratuavary, Munzur University. The cultured *D. polymorpha* individuals were brought alive to the Toxicology Research Laboratory and adapted to these conditions for 30 days.

Adaptation of D. polymorpha to laboratory conditions

D. polymorpha were selected from healthy individuals of similar size. Laboratory temperature and lighting was controlled. In the illumination, a photoperiod of 12:12 light:dark was applied. Temperature was set 19±0.5 °C, 22±0.5 °C and 25±0.5 °C with thermostat at all experimental stages. Also external filter was used for water circulation in stock aquariums where the test organism is kept. Nutrition and mobility of living organisms has been observed during adaptation.

Experimental design

The following 12 test groups at the ratios of 1/20, 1/10 and 1/5 of LC_{50} values created at different tempatures 19, 22 and 25 °C. Ten *D. polymorpha* individuals were used in all groups.

Control group, organisms not exposed to any substance at 19°C. Group A, organisms were exposed to λ-cyhalothrin at 1/20 of the LC_{50} value at 19 °C. Group B, organisms were exposed to λ-cyhalothrin at 1/10 of the LC_{50} value at 19 °C. Group C, organisms were exposed to λ-cyhalothrin at 1/5 of the LC_{50} value at 19 °C. Control group, organisms not exposed to any substance at 22 °C. Group D, organisms were exposed to λ-cyhalothrin at 1/20 of the LC_{50} value at 22 °C. Group E, organisms were exposed to λ-cyhalothrin at 1/10 of the LC_{50} value at 22 °C. Group F, organisms were exposed to λ-cyhalothrin at 1/5 of the LC_{50} value at 22 °C. Control group, organisms not exposed to any substance at 25 °C. Group G, organisms were exposed to λ-cyhalothrin at 1/20 of the LC_{50} value at 25 °C. Group H, organisms were exposed to λ-cyhalothrin at 1/10 of the LC_{50} value at 25 °C. Group I, organisms were exposed to λ-cyhalothrin at 1/5 of the LC_{50} value at 25 °C.

Supernatant preparation

For supernatants preparation, the shells of *D. polymorha* organisms were opened by cutting the adductor muscles with the help of scapula, scalpel and spatula and then 0.5 g of *D. polymorpha* individuals were weighed and 1/5 w/v in PBS buffer containing 5 µL of protease inhibitor cocktail and homogenize using a homogenizer with ice. These homogenized samples were placed in a cooled centrifuge at 17000 g for 15 minutes. The obtained supernatants were stored in a deep freezer at -80 °C.

Determination of biochemical response

In our study, AChE, SOD, CAT enzymes and GSH, MDA levels were used to determine the biochemical response. GSH, SOD, CAT and MDA kits were purchased from CAYMAN and

AChE kits used in the study were purchased from CUSABIO company (Catalog numbers GSH: 703002, SOD: 706002, CAT: 706002 AChE: CSB-E17001Fh, MDA: 10009055).

GSH assay kit utilizes a carefully optimized enzymatic recycling method, using glutathione reductase, for the quantification of GSH. The sulfhydryl group of GSH reacts with

DTNB (5,5'-dithio-bis2-(nitrobenzoic acid), Ellman's reagent) and produces a yellow colored 5-thio2-nitrobenzoic acid (TNB). Measurement of the absorbance of TNB at 405-414 nm provides an accurate estimation of GSH in the sample.

Superoxide dismutase assay kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical measured in change in absorbance per minute at 25°C and pH 8.0.

Catalase Assay Kit utilizes the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H2O2. The formaldehyde produced is measured colorimetrically with 4-amino-3-hydrazino5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color.

TBARS Assay Kit provides a simple, reproducible, and standardized tool for assaying lipid peroxidation. The MDA-TBA adduct formed by the reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions is measured colorimetrically at 530-540 nm or fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm.

AChE assay kit employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for AChE has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any AChE present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for AChE is added to the wells. The color development is stopped and the intensity of the color is measured.

Statistical analysis

Statistical analysis were carried out using SPSS 24.0 statistics program. The LC_{50} value of λ-cyhalothrin Insecticide in *D. polymorpha* was calculated using Probit analysis. The statistical difference between different groups was determined by the Duncan's multiple range test. The difference between the application times (24 and 96 hours) was determined by independent t-test.

Results

LC_{50} values of λ-cyhalothrin were determined with range determination tests in *D. polymorpha* individuals for 19, 22 and 25 °C and the results are shown in Table 1.

Table 1: LC_{50} values of λ-cyhalothrin for *D. polymorpha* individuals at different temperatures[1]

Temperature (°C)	LC_{50} value (mg/L λ-cyhalothrin)
19	2.23 ± 0.27^{b}
22	2.61 ± 0.1^{0ab}
25	2.71 ± 0.21^{a}

[1]All data are presented as the mean ± standard error of the mean (SEM). Different letters on the means indicate a statistically significant difference between different temperatures

AChE activity increased at 19 °C at the end of the 24th hour in groups A and B when decreased as statistically significant in the group C compared the control group. AChE activity decreased in group D and increased in groups E and F at the end of 24th hour at 22 °C. An increase was detected at the end of the 96th hour at 22 °C. A statistically significant decrease was detected in group I at the end of the 24th hour at 25 °C in AChE activity but no statistically significant changes were observed at the end of 96th hour. Statistical differences were found in the groups B, D, E, H and I when application times compared (Figure 1).

A statistically significant increase was observed at 19 °C and 22°C in CAT activities in all application groups when compared to the control group at 24 and 96 h ($p<0.05$). A statistically significant increase was observed at 25 °C in CAT activities in groups G, H and I when compared to the control group during 24 h ($p<0.05$) but decreased during 96 hours. Statistical differences were found in the groups A, B, C, E, F, G, H, I when application times compared ($p<0.05$) (Figure 2).

A statistically significant decrease was observed in SOD activities in all application groups at 19, 22 and 25 °C when compared to the control group during 24 h ($p<0.05$). A statistically significant decrease was observed in the groups A, B, D, E, F, G and H at 19 °C, 22 °C and 25 °C when compared to the control group during 96 h ($p<0.05$). A statistical difference was found in the groups H and I when application times compared ($p<0.05$) (Figure 3).

GSH levels were decreased in *D. polymorpha* exposed to λ-cyhalothrin at 19, 22 and 25 °C for 24 and 96 hours ($p<0.05$). A statistical difference was found in the groups H and I when application times compared ($p<0.05$) (Figure 4).

No significant changes were found in MDA levels at 19 °C compared to control for 24 hours ($p< 0.05$) but a statistically

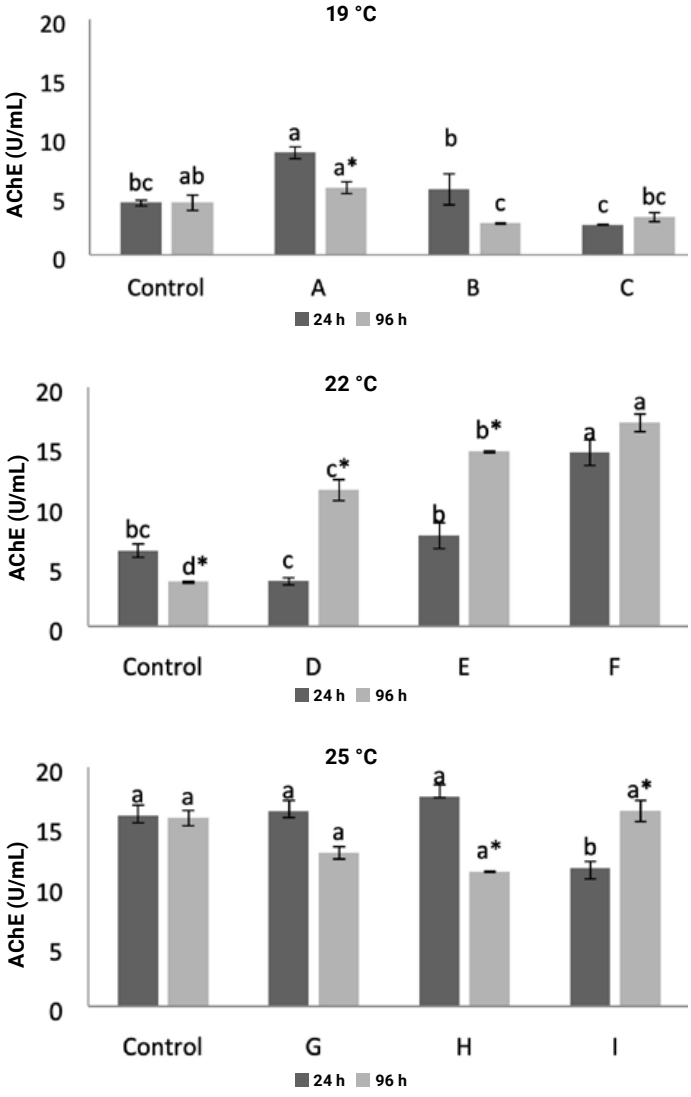

Figure 1: Changes in AChE enzyme activities in *D. polymorpha* exposed to λ-cyhalothrin pesticide at 19, 22 and 25 °C for 24 and 96 hours. Different letters on the columns indicate a statistically significant difference between different application doses, and the * on the columns indicate a statistically significant difference between the application times (p<0.05)

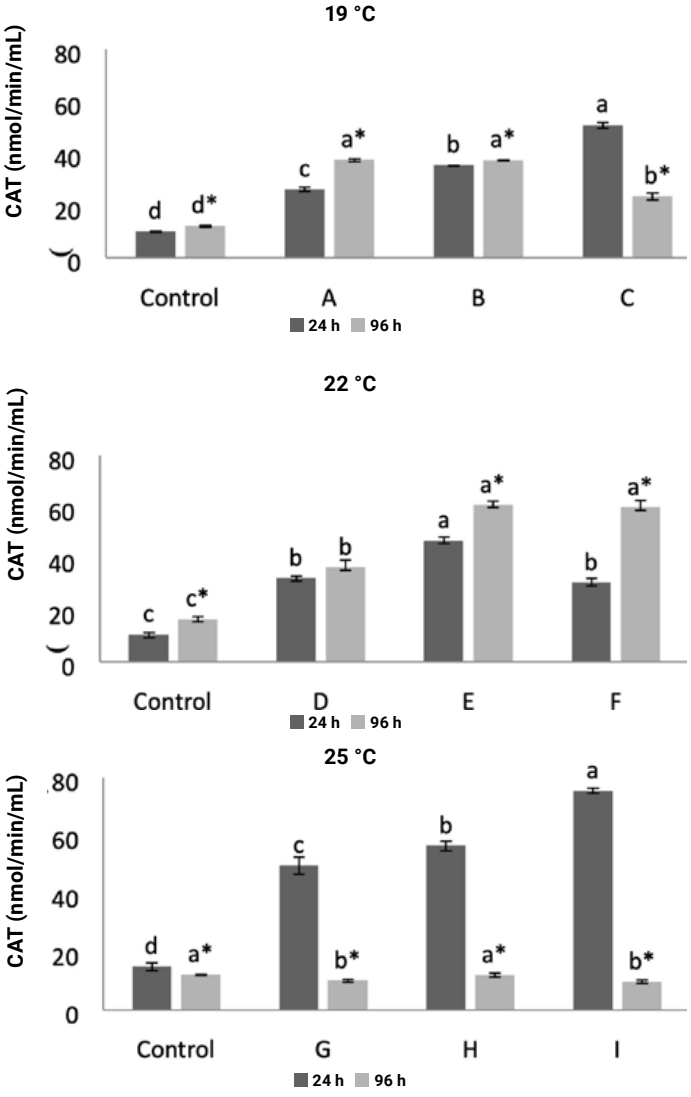

Figure 2: Changes in CAT enzyme activities in *D. polymorpha* exposed to λ-cyhalothrin pesticide at 19 °C, 22 °C and 25 °C for 24 and 96 hours. Different letters on the columns indicate a statistically significant difference between different application doses, and the * on the columns indicate a statistically significant difference between the application times (p<0.05)

significant increase was observed in groups B and D when compared to the control group at 96th hours (p<0.05). MDA levels were increased significantly in *D. polymorpha* exposed to λ-cyhalothrin at 22 °C and 25 °C for 24 and 96 hours (p<0.05). A statistical difference was found in the groups B and I when application times compared (p<0.05) (Figure 5).

Discussion

λ-cyhalothrin is highly toxic to many aquatic organisms including fish, invertebrates and amphibians (26). λ-cyhalothrin is discharged directly water resources through agricultural use and forest spraying procedures and accumulates in sediment (27). Temperature has various effects on a wide range of physiological effects, including bioavailability, adsorption, elimination, and relative toxicity of chemicals in aquatic poikilotherms (28). Temperature can affect the physico-chemical behavior (decomposition, evaporation, transport, transfer and accumulation) of chemicals (29). Temperature can directly affect the mobility of the chemicals and change the uptake rate of these chemicals by aquatic organisms. Due to the rapid spread of chemicals at high temperatures, the rate of uptake of these chemicals into the organism increases. This results in faster reaching the toxicological threshold for the chemical. Temperature can also affect the toxicity of a chemical by affecting its degradation. In a study conducted Tasmin et al. (2014) it was shown that the herbicide diuron has lower toxicity at higher temperatures due to increased chemical degradation/volatility rates at higher temperatures in green algae *Pseudokirchneriella ubcapitata* (30). Similarly, damselfly *Ischnura elegans* exposed to the chlorpyrifos had lower toxicity at 24 °C versus 20 °C, as less toxic compounds were formed at a higher biodegradation rate (28). It has

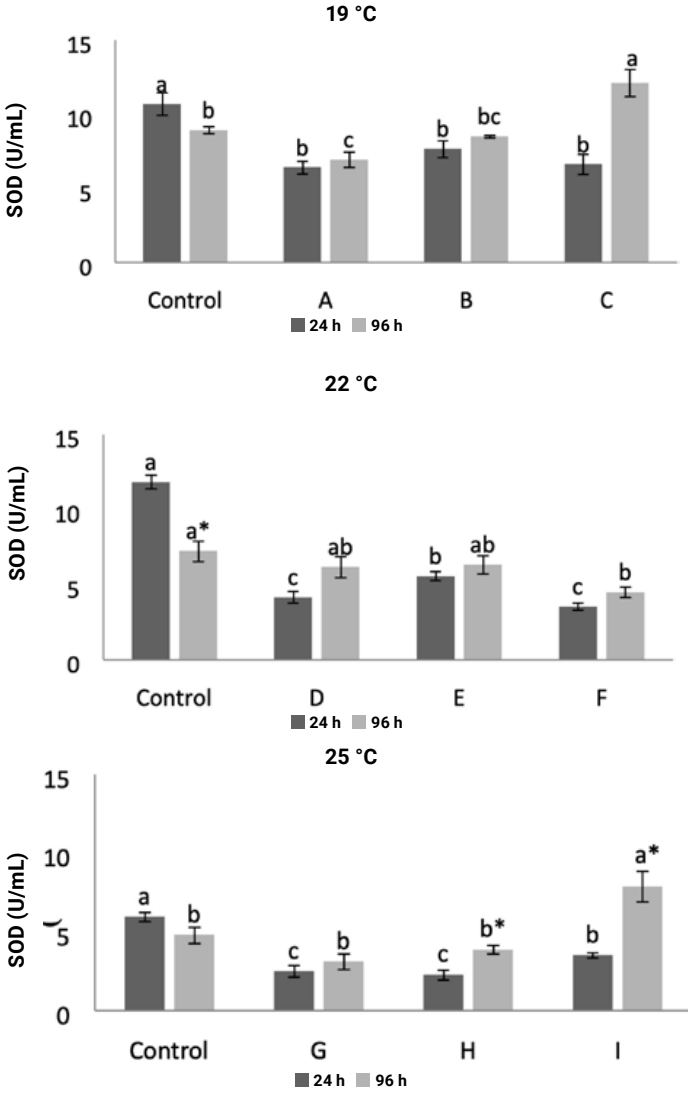

Figure 3: Changes in SOD enzyme activities in *D. polymorpha* exposed to λ-cyhalothrin pesticide at 19 °C, 22 °C and 25 °C for 24 and 96 hours. Different letters on the columns indicate a statistically significant difference between different application doses, and the * on the columns indicate a statistically significant difference between the application times (p<0.05)

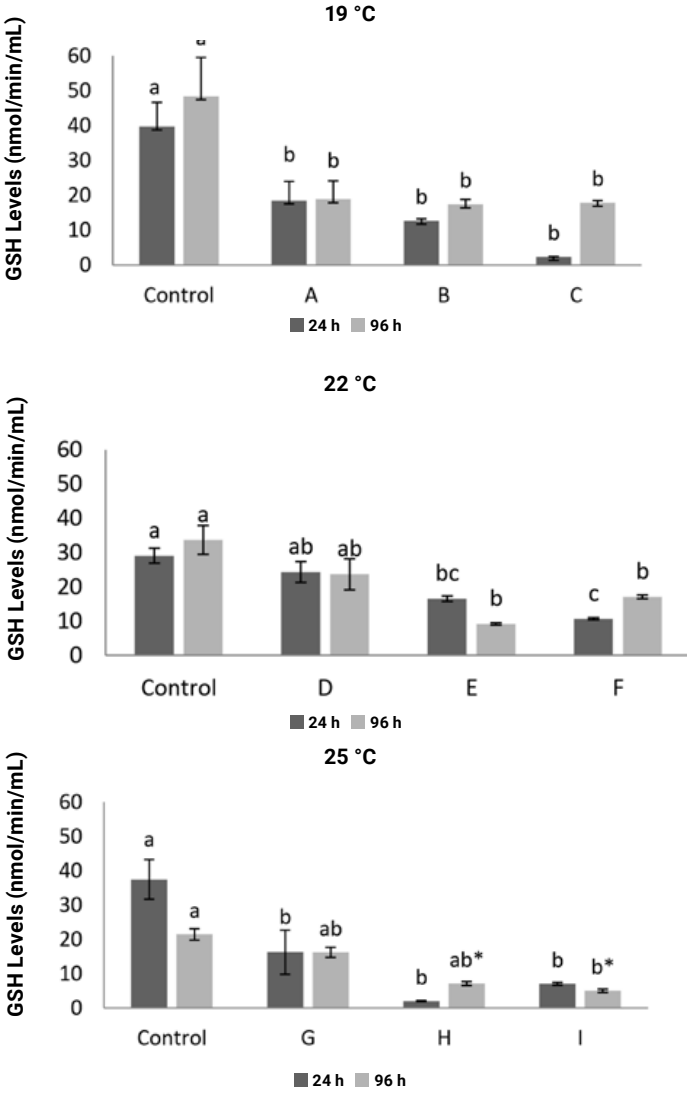

Figure 4: Changes in GSH levels in *D. polymorpha* exposed to λ-cyhalothrin pesticide at 19°C, 22 °C and 25 °C for 24 and 96 hours. Different letters on the columns indicate a statistically significant difference between different application doses, and the * sign on the columns indicate a statistically significant difference between the application times (p<0.05)

been observed that pyrethroids are more toxic in winter than in summer, and the 96-hour LC_{50} values can change approximately tenfold at 10, 15 and 20ºC (31). The zebra mussel is a creature whose body temperature changes according to environmental temperature fluctuations. The metabolic rate of zebra mussels can be affected by various factors, including temperature. The toxicity of pyrethroids was found to increase with decreasing temperature (32). Garcia et al. (2011) evaluated the effects of λ-cyhalothrin insecticide on earthworms using acute and chronic toxicity tests modified for tropical conditions (20 and 28 °C) and on two strains of compost worm (temperate and tropical). It has been observed that the effects of λ-cyhalothrin in soils do not change much at two temperatures. In tropical soils at high temperatures, the effects differ up to ten times. In present study, it has been observed that different temperature treatments have different effects on the toxic effects of λ-cyhalothrin (33). These findings are consistent with the results of the current study.

In the literature, no study was found that studied the toxic effects of λ-cyhalothrin on *D. polymorpha* at different temperatures. Göksu et al. (2015) investigated the acute toxic effects of λ-cyhalothrin on *Oreochromis niloticus* (L., 1754) offspring in their study. λcyhalothrin 24-hour LC_{50} value was 6.80±0.63 μgL^{-1} (34). The LC_{50} value for *Channa punctatus* was 6.88 μgL^{-1} (35). Chatterjee et al., (2021a) showed that 96 h LC_{50} value of λ cyhalothrin to *Tubifex tubifex* are 0.13 mg L^{-1}(36). Chatterjee et al., (2021b) evaluate the toxic effects of λ cyhalothrin on the common carp, *Cyprinus carpio L.* The results depicted that 96 h LC_{50} value of λ cyhalothrin to the fish was 1.48 µg L^{-1} (37). In a study conducted by Bibi et al. (2014), the 96 h LC_{50} value of Karate (λ-Cyhalothrin as an active ingredient) was found to be 0.160 µL L^{-1} (38). The LC_{50} values of λ-Cyhalothrin were

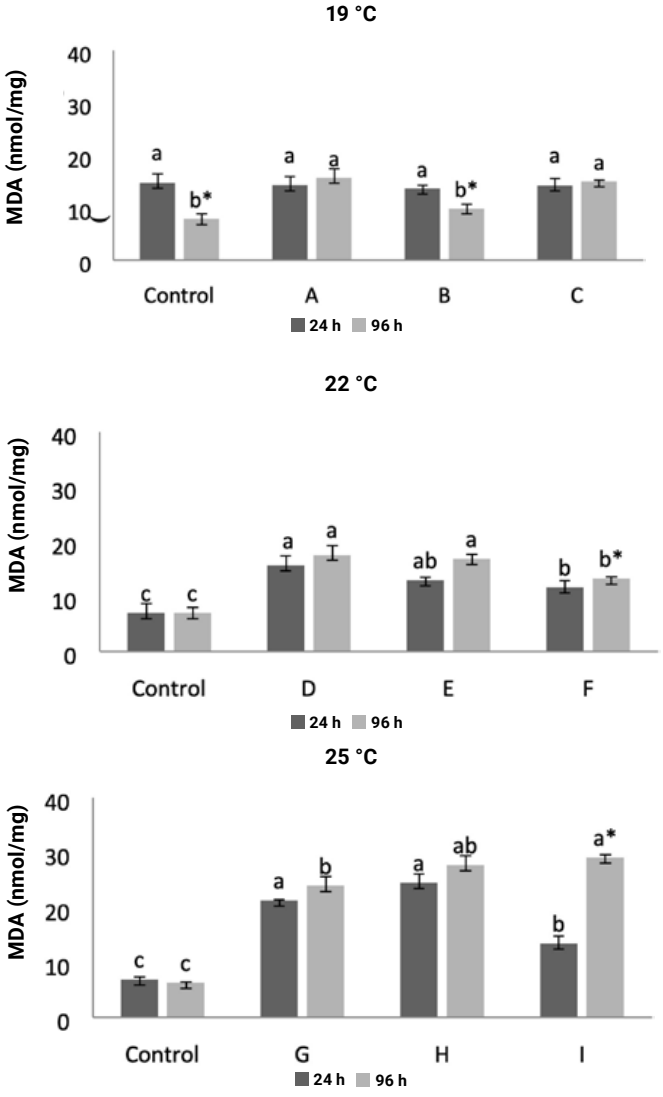

Figure 5: Changes in MDA levels in *D. polymorpha* exposed to λ-cyhalothrin pesticide at 19°C, 22°C and 25°C for 24 and 96 hours. Different letters on the columns indicate a statistically significant difference between different application doses, and the * sign on the columns indicate a statistically significant difference between the application times (p<0.05)

0.571, 0.380, 0.337 and 0.325 ppm at the exposure time of 24, 48, 72 and 96 h, respectively for African catfish *Clarias gariepinus* (39). In present study, LC_{50} values of λ-cyhalothrin at 19 °C, 22 °C, 25 °C is 2.23 ± 0.27, 2.61± 0.10, 2.71±0.21 respectively. In present study, LC_{50} values increased as statistically significant with increasing temperature.

The SOD-CAT antioxidant system scavenges free radicals and thus fights against oxygen damage (40). Chatterjee et al. (2021b) evaluated the toxic effects of λ-cyhalothrin on *Cyprinus carpio L*. It was observed that GST exhibited a significant initial increase followed by a decrease, a decrease in CAT, SOD levels, and a significant increase in MDA levels in liver and gill due to increased λ-cyhalothrin concentrations (37). In another study conducted by Chatterjee et al. (2021a), initial induction followed by a subsequent reduction in SOD, GSH, and GST were found in *T. tubifex* exposed to non-lethal concentrations of λ-cyhalothrin (0.013 and 0.026 mg L^{-1}) for 14 days. It has been also shown to cause an induction in MDA and CAT over the exposure period (36). Okechukwu and Auta, (2007) investigated the impact of long-term exposure to waterborne λ--cyhalothrin on *Clarias gariepinus* through changes of selected biochemical parameters. *C. gariepinus* was exposed to 0.0004, 0.0008 and 0.0016 mg L^{-1} for 8 weeks. The alterations in all parameters were significantly dose and time dependent (41). Ezenwosu et al., (2021) investigated the effect of λ-cyhalothrin on oxidative stress in *Clarias gariepinus*. On the 7th day, CAT activity increased, on the 14th day; It was determined that SOD activity increased and there was a significant increase in all parameters except SOD on the 21st and 28th days (42). Koç and Akçay (2018) investigated the effects of λ-cyhalothrin on *Capoeta capoeta* (Guldenstaedt 1773) caught from Kars Brook by biochemical and molecular methods. When the expression levels of CAT and SOD enzymes were investigated by RT-PCR method, it was determined that there was an increase in SOD and CAT enzyme expression levels compared to the control group (43). In present study, it was also shown that λ-cyhalothrin application caused a decrease in SOD enzyme activity in *D. polymorpha*, and the application time and temperature changed the enzyme activity. CAT enzyme activity increased depending on the application dose. As the dose increased, an increase in enzyme activity was also observed. It was observed that the temperature at which the highest increase occurred at 25 °C increased the toxic effect. At 25 °C, which is the highest application temperature, an inhibition of CAT enzyme activity occurred again. These changes in SOD and CAT enzyme activities are indicative of a defense mechanism developed by the model organism against oxidative stress induced by a polluting, λ-cyhalothrin. The toxic effect gradually increased with increasing dose and temperature.

It has been suggested that MDA, a highly reactive bifunctional molecule, is an end product of membrane lipid peroxidation, one of the pesticide-induced toxicity mechanisms (20). Kumar et al. (2012) showed that *Channa punctatus* exposed to pyrethroid insecticides λcyhalothrine for 96 hours significantly increased LPO levels in different organs such as brain, liver, kidney, gill and muscle. The remarkable increase in the LPO indicates strong stress inducing potential of λ-cyhalothrin in fishes (12). In present study, MDA levels were increased significantly in *D. polymorpha* exposed to , λ-cyhalothrin at 22 °C and 25 °C for 24 and 96 hours (p<0.05). Serdar et al. (2021) investigated the effect of temperature on the freshwater amphipod *Gammarus pulex* (l., 1758). It was determined that the MDA level increased with the increase in temperature and Cd concentration. The increased MDA levels reflect the increase LPO found in the present investigation may have resulted from an increase of free radicals as a result of stress condition generated by pesticide exposure (44).

Literature search showed that the toxicity of pyrethroids increases as the temperature decreases (45). The use of

high GSH for conjugation and/or the use of GSH as an antioxidant to neutralize free radicals may cause GSH content depletion (46). In this study, a general decrease was found in GSH levels due to the administration of λ-cyhalothrin. Especially at the end of the 96th hour, the maximum decrease was found at 19 °C. A steady decrease in concentration was observed. It has been observed that this decrease in GSH levels is an adaptive response developed by zebra mussel *D. polymorpha* to cope with the oxidative stress that occurs due to λ-cyhalothrin application, and temperature affects the oxidative response. Decreased temperature increased the severity of the response to oxidative stress.

Inhibition of AChE activity causes decreased cellular metabolism, induce deformities of the cell membrane, and disturbs of metabolic and neural activity, ionic refluxes and differential membrane permeability (47, 48). Razik and El-Raheem (2019) the activity of AChE activity decreased after treated with LC_{30} and LC_{50} of the indoxacarb (49). Vieira and Martinez (2018) evaluated the acute effects of λ-cyhalothrin in juveniles of the teleost *Prochilodus lineatus* exposed for 96 h to four concentrations (5, 50, 250 and 500 ng L^{-1}). They observed that AChE activity decrease in the muscles of fish at all concentrations (50). Decremented AChE level exposed to λ-cyhalothrin probably leads to excessive acetylcholine accumulation at the synapses and neuromuscular junctions, resulting in hyperstimulation of the nervous system that causes behavioral changes and eventually death of the organism (51). In the study conducted by Bibi et al. (2014) fry of *Cyprinus carpio* were exposed to 10% (0.16 μL L^{-1}) and 20% (0.032 μL L^{-1}) lethal concentration of Karate (λ-Cyhalothrin as an active ingredient) and observed the effects on total protein content and AChE activity in brain, liver and muscle tissues. AChE activity in different tissues of *C. carpio* decreased in concentration dependent manner and showed tissue specific pattern (38). In this present study, while a general inhibition was observed in AChE levels, this inhibition was observed to be more especially in the groups administered high-dose λcyhalothrin. Also, as the application times increase, it was seen that the inhibition levels increase. It has been observed that different temperatures cause changes in enzyme levels. It has been suggested that changes in exposure temperature may alter the binding affinities of lipophilic toxins within the lipid-rich neural fat body sheath associated with insect nervous systems, thereby interfering with ion channel activation and membrane. Neurons associated with the neural membrane may be affected by temperature, which may lead to disruption of the permeability mechanism (52). In present study, AChE levels were inhibited especially in the groups administered high-dose λ-cyhalothrin. It was also found that the inhibition levels increased depending on the application times.

Conclusions

It was determined that λ-cyhalothrin has a toxic effect on *D. polymorpha* and this toxic effect increases depending on the temperature. In our study, it was concluded that the D. polymorpha model organism and some of its biochemical parameters (AChE, CAT, SOD, GSH and MDA) are suitable biomarkers for revealing the effect of temperature variable on toxic response. It has also been shown that biochemical parameters such as SOD, CAT, AChE activities and GSH, TBARS levels used are suitable biomarkers for the evaluation of the toxic effects of λ-cyhalothrin.

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Vrednotenje vpliva temperature na toksičnost Lambda-cihalotrina v modelnem organizmu Dreissena Polymorpha z uporabo biokemijskih označevalcev

N. C. Yildirim, O. Serdar, Z. Ketenalp

Izvleček: Zaradi naraščajočih sprememb podnebja je pomembno ugotavljati, ali temperatura vpliva na razmerje med odmerkom in odzivom organizmov na nekatere snovi. Zato je bil namen te študije prikazati vpliv spremembe temperature na toksični odziv modelnega organizma Dreissena polymorpha in nekaterih njegovih bioloških oiznačevalcev. V ta namen smo s komercialnimi testi ELISA merili koncentracijo acetilholinesteraze (AchE), katalaze (CAT), superoksid dismutaze (SOD), glutationa (GSH) in malondialdehida (MDA) v organizmu Dreissena polymorpha, izpostavljenim subletalnim odmerkom λ-cihalotrina pri različnih temperaturah. V skupinah, izpostavljeni λ-cihalotrinu, se je statistično značilno povečala vsebnost MDA in zmanjšala vsebnost GSH. Ravni AChE so bile znižane zlasti v skupinah, ki so bile izpostavljene visoki koncentraciji λ-cihalotrina. Ugotovili smo tudi, da je bila rast inhibicije odvisna od časa aplikacije. Medtem ko se je aktivnost encima SOD zmanjšala, se je aktivnost encima CAT povečala glede na koncentracijo izpostavljenosti. Ugotovili smo, da različna temperatura različno vpliva na toksičnost λ-cihalotrina. λ-cihalotrin povzroča oksidativni stres in nevrotoksičnost, toksičnost λ-cihalotrina pa se spreminja glede na temperaturo.

Ključne besede: λ-cihalotrin; D. polymorpha; oksidativni stres; nevrotoksičnost; temperatura

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Original Research Article

Effects of Irisin on the Reproductive System of Obese Female Rats Induced by a High-fat Diet

Key words

irisin;
obesity;
hormones;
ovary;
apoptosis;
female reproduction

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Abstract: Obesity is becoming more common all across the world, causing a variety of health problems, including reproductive disruption. Although the novel, exercise-induced hormone irisin may affect the hypothalamus-pituitary-gonadal axis and reproductive function control, its impact on obesity-induced damage to the female reproductive system is not fully known. Hence, this study aimed to investigate the potential effects of irisin on reproductive hormones and reproductive organs in female rats with obesity induced by a high-fat diet. Forty female rats were divided into four groups: control, irisin, obese, and obese+irisin (n = 10 in each group). After simulating a high-fat diet-induced obesity model (via 60% kcal fat for 12 weeks) in the obese and obese+irisin groups, irisin (100 ng/kg/day via mini-osmotic pumps for about 28 days) was administered subcutaneously to the irisin and obese+irisin groups. Results showed that subcutaneous irisin perfusion increased serum luteinizing hormone (LH), the LH to follicle-stimulating hormone (FSH) ratio (LH/FSH), and progesterone levels while decreasing the histopathological damage in the ovaries of obese rats. On the other hand, endogenous irisin serum concentrations were similar in lean female rats and obese female rats with reproductive disorders. These results suggest that irisin may affect the reproductive axis in obese female rats. An increase in serum LH levels, which trigger ovarian steroidogenesis, and reducing histopathological changes in ovarian tissue could contribute to this effect.

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Introduction

The prevalence of obesity has rapidly increased worldwide, and obesity causes several health problems, including reproduction. Obesity directly or indirectly negatively affects the hypothalamus-pituitary-ovarian (HPO) axis, resulting in various reproductive disorders by causing hormone imbalances and ovulatory dysfunction. Additionally, it is also known that obesity may have a direct effect on ovarian function, independent of the HPO axis (1, 2). Studies in humans and rodents have shown that obesity-related ovarian dysfunction includes abnormalities in folliculogenesis and ovulation, irregular estrous cyclicity, and depletion of ovarian reserve (3).

Lifestyle modifications, including a healthy diet and exercise, are successful options for treating women with reproductive dysfunctions. Specifically, exercise has a protective effect against obesity-induced impairment in the female reproductive system. Exercise, for example, has been shown to improve menstrual cyclicity, ovulation, and pregnancy rates in obese anovulatory women (4, 5). Similarly, in obese polycystic ovary syndrome (PCOS) rats fed a high-fat diet, swimming training also appeared to recover ovarian morphology indexes such as the numbers of antral follicles and corpora lutea (6).

Irisin was recently discovered as a new hormone-like myokine released into the circulation in response to physical exercise. It is produced by cleavage of its precursor, fibronectin type III domain containing 5 (FNDC5). Following secretion, irisin promotes the browning of white adipocytes and the expression of uncoupling protein 1 (UCP1), leading to enhanced UCP-1-mediated thermogenesis and increased energy expenditure (7, 8). Irisin has a potential role in mammalian growth and regulation of the reproductive axis (9-11). It is documented that irisin deficiency is associated with disordered endocrine metabolism, poor growth, and decreased fertility in female mice (9). Interestingly, it is known that irisin can cause different effects on reproductive function depending on gender in the rat model, such as exhibiting androgenic activity in males and causing reproductive disorders in females (12). Furthermore, recent studies have shown that irisin improves antidepressant-induced sexual dysfunction and obesity-related reproductive disorders. Accordingly, it has been revealed that irisin may mediate the effects of exercise on the reproductive system and that irisin and exercise may also have similar effects on reproductive potential (13-16). However, there are not enough studies on the possible potent effects of the irisin hormone on reproductive system disorders caused by obesity in female rats.

This study was performed to investigate the effects of exogenous irisin on serum levels of reproductive hormones and insulin and on the reproductive organs in obese female rats. Furthermore, endogenous irisin levels were measured in obese female rats to determine whether irisin levels are associated with obesity-induced changes in sex hormones and reproductive organs.

Material and Methods

Animals and diets

All animal experiments were carried out in accordance with the governmental guidelines for the care and use of laboratory animals at Firat University and approved by the Animal Experimental Ethics Committee of Firat University (31.01.2018, number 19). Sprague-Dawley female rats (2-3 months old, 200-250 g) were obtained from the Firat University Experimental Research Unit (Elazig, Turkey). Animals were housed 3-4 per cage and kept in a 12 h light/12 h dark cycle (light on 07:00-19:00), under standard conditions (21 ± 1 °C temperature and 50-60% humidity), with *ad libitum* access to water and food. Forty rats were randomly allocated to four treatment groups as follows: control (control group with rats subjected to vehicle treatment), irisin (irisin-treated rats), obese (obesity-induced rats), and obese+irisin (irisin-treated obesity-induced rats) (n = 10 rats per group). All rats in the control and irisin groups were fed with standard commercial rat food (Korkuteli Yem Gıda Santic A.Ş., Antalya, Turkey), while all rats in the obese and obese+irisin groups were fed a high-fat diet (D12492, Research Diets, 60% kcal fat) for 16 weeks. After 12 weeks of high-fat diet exposure, the induction of obesity in both obesity-induced treatment groups was confirmed by measuring the Lee index, which is used for experimental validation of obesity as described previously (16-18).

Continuous administration of irisin

After 12 weeks of diet exposure (i.e., after the establishment of obesity due to the high-fat diet), all rats in the control, irisin, and obese+irisin groups were anesthetized with a mixture of ketamine (6 mg/kg) and xylazine (5 mg/kg) anesthetics, then a mini-osmotic pump (Alzet, Model 2004; Durect Corp., Cupertino, CA) was subcutaneously implanted between the scapulae of each animal under sterile conditions. Alzet mini-osmotic pumps were filled with either deionized water (vehicle) for the control group or irisin (SRP8039, Sigma-Aldrich; dissolved in deionized water to deliver at a dose of 100 ng/kg/day) for both irisin and obese+irisin groups as previously described (18). The Alzet model 2004, with a reservoir volume of 200 µL, infused deionized water or irisin at a flow rate of 0.25 µL/h for about 28 days.

Sample collections

The serum hormone levels of female rats fluctuate greatly depending on the estrous cycle, and therefore, all rats were in the same estrous cycle phase (diestrus) to determine the comparability of hormone levels at the end of the experiment. On days 25-28 of deionized water or irisin perfusion, all rats in the diestrus phase as determined by vaginal smears were sacrificed by decapitation at the light phase between 17:00 h and 19:00 h. After decapitation, trunk blood was immediately collected for a serum-based enzyme-linked immunosorbent assay (ELISA) and centrifuged (4500 rpm; 4 oC; 5 min) to obtain serum samples, which were stored at -20 oC. In addition, the uterine horns and ovaries were immediately excised and cleaned of fat, and their wet weights were measured and expressed as mg/100 g body weight (BW). The final body weight of each animal was measured just before decapitation. The mid-portion of the uterine horns and all of the ovaries were fixed in a 10% formaldehyde solution for histopathological investigations. The serum and reproductive organs used in this study belonged to the animals used in the previous study (18).

Hormone measurements

Commercial rat ELISA kits were purchased from Elabscience Biotechnology Inc. (Texas, USA) and Enzo Life Sciences (Switzerland). They were used to assess in duplicate the follicle-stimulating hormone (FSH; E-EL-R0391), luteinizing hormone (LH; ENZ-KIT107), 17β-estradiol (E2; ADI-900-008), progesterone (ADI-900-011), testosterone (ADI-900-065), insulin (E-EL-R3034), and irisin (FNDC5, E-EL-R1104) serum levels according to the manufacturer's instructions

by using an ELISA microplate reader (Multiskan FC, Thermo Scientific, USA). Serum FSH, LH, E2, progesterone, testosterone, and insulin levels were quantified in all groups. On the other hand, serum irisin levels were measured only in the control and obese groups to determine whether obesity-induced damage to the female reproductive system is related to irisin levels.

Histopathological examinations

For light microscopic evaluation, formalin-fixed uterus and ovary tissues were dehydrated through an increasing alcohol series (70%, 80%, 96%, and 100%) and then embedded in paraffin wax. Afterward, sections with a thickness of 5 µm were cut from uterine and ovarian tissues and stained with hematoxylin and eosin (H&E) and/or Masson trichrome staining. All of the uterine and ovarian tissue sections were evaluated and photographed by a blinded examiner using a Leica DM500 light microscope (DFC295; Leica, Wetzlar, Germany). At 20x magnification, twenty random fields from a section of each ovary and uterus were examined and/or scored.

The follicles (primordial, primary, secondary, and Graaf follicles) and corpus luteum in twenty random fields of each ovarian section were counted. Ovarian follicles were classified according to the morphologic criteria as described by Artaş et al. (2018) (19). Histopathological examination of the uterine and ovarian tissue damage was carried out by a thorough qualitative histologic examination, and fibrosis pathology was semi-quantitatively scored (0, no fibrosis; 1, low fibrosis; 2, intermediate fibrosis; 3, severe fibrosis) (12, 20).

TUNEL Assay

The apoptosis in ovarian tissues was evaluated by the terminal deoxynucleotidyl transferase-mediated deoxyuridine-biotin nick end labeling (TUNEL) method. For the detection of apoptotic cells, the ApopTagPlus Peroxidase in Situ Apoptosis Detection Kit (Chemicon, Lot: 3006560, USA) was used according to the manufacturer's instructions. In the evaluation of the TUNEL assay, the nuclei of healthy cells were blue, and the cells with stained brown nuclei were considered apoptotic cells. A total of 200 cells were counted in randomly selected areas under light microscopy at 20x magnification. Accordingly, the apoptotic index (%) was calculated as the ratio of the number of TUNEL-positive cells to the total number of cells (21).

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) and were analyzed using the SPSS 22.0 software. Before analysis, the normality of all data was verified using the Shapiro-Wilk test. For multiple comparisons between groups, a one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test was utilized. Comparisons between the control and obese groups were evaluated by an unpaired student t-test (irisin levels). $p<0.05$ was considered statistically significant.

Results

Serum sex hormone levels

Serum FSH, LH, LH/FSH ratio, E2, progesterone, and testosterone levels in all experimental groups are shown in Fig. 1. When compared to the control group, serum FSH levels decreased significantly in the irisin group ($p<0.05$) although they remained the same in both the obese and obese+irisin groups (Fig. 1A). Serum LH levels were found to increase significantly in the obese and obese+irisin groups due to the high-fat diet ($p<0.001$), but no obvious changes were observed in the irisin group. Additionally, compared to the obese group, serum LH levels in the obese+irisin group significantly increased depending on subcutaneous administration of irisin ($p<0.001$) (Fig. 1B). The LH/FSH ratio was significantly increased depending on the decreased serum FSH levels and unchanged serum LH levels in the irisin group ($p<0.01$, Fig. 1C). As a result of increased serum LH concentration due to the high-fat diet, it was determined that LH/FSH ratios increased in both obese and obese+irisin groups ($p<0.05$ and $p<0.001$, respectively, Fig 1C). Moreover, compared with the obese group, the LH/FSH ratio of rats in the obese+irisin group was significantly increased depending on irisin exposure ($p<0.001$, Fig. 1C).

As seen in Fig. 1D, serum E2 levels were reduced in obese rats when compared to control rats ($p<0.05$). Serum progesterone levels were significantly increased due to subcutaneous irisin perfusion in the irisin and obese+irisin groups as compared to the control group ($p<0.01$). This increase in the obese+irisin group was also found to be significant compared to the obese group ($p<0.01$) (Fig. 1E). In comparison with the control group, serum testosterone levels were substantially increased in the obese+irisin group ($p<0.01$). On the other hand, it was determined that serum testosterone levels were not affected by subcutaneous irisin perfusion or high-fat diet exposure alone (Fig. 1F).

Serum insulin and irisin levels

Continuous administration of irisin, high-fat diet exposure, or both continuous administration of irisin and high-fat diet exposure in female rats did not cause a change in serum insulin levels compared with female rats treated with a vehicle (Fig. 2A). As shown in Fig. 2B, there was no significant difference in the serum irisin levels in female rats exposed to a high-fat diet in comparison to the vehicle-treated female rats.

Reproductive organ weights

Irisin perfusion had no significant effect on body weight in both lean and obese rats, as determined in our previous study (18). As shown in Table 1, there was a significant reduction in the uterine wet weights normalized for body weights in both irisin and obese+irisin groups compared to the control group ($p<0.05$ and $p<0.001$, respectively). When compared to the control group, high-fat diet exposure significantly decreased reproductive organ weights in female rats ($p<0.05$ for ovarian wet weights normalized to body weights and $p<0.01$ for uterine wet weights normalized to body weights). Also, it was determined that subcutaneous irisin perfusion had a statistically insignificant effect on the

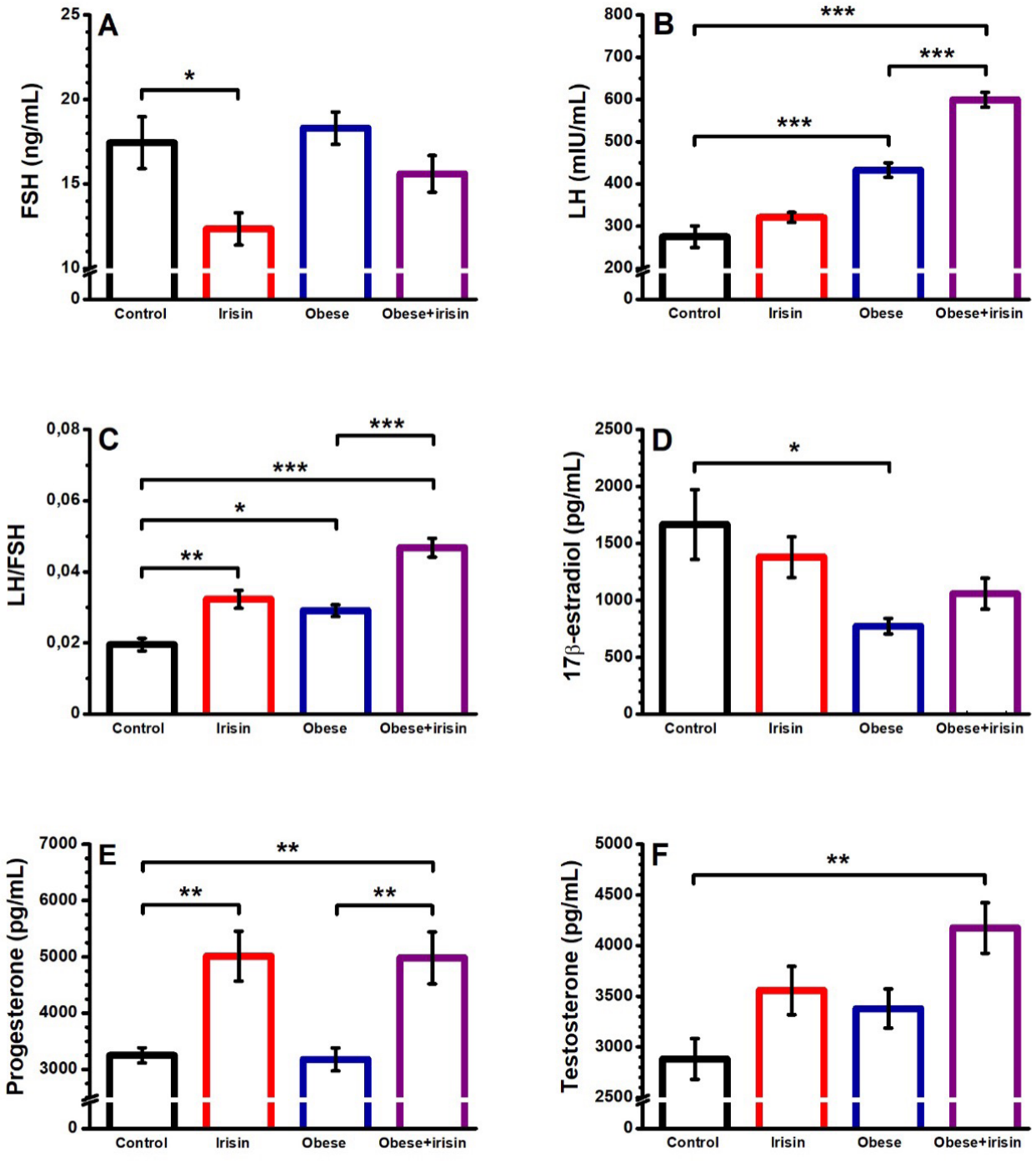

Figure 1: Effects of subcutaneous irisin perfusion and/or high-fat diet-induced obesity on serum reproductive hormones in female rats. A) Serum FSH levels. B) Serum LH levels. C) LH/FSH ratio. D) Serum 17β-estradiol levels. E) Serum progesterone levels. F) Serum testosterone levels. Data were expressed as mean ± SEM. *p<0.05, **p<0.01, and ***p<0.001 (one-way ANOVA followed by the Tukey's *post-hoc* test, n = 10 in each group). FSH: follicle-stimulating hormone, and LH: luteinizing hormone

decreased reproductive organ weights of high-fat diet-induced obese rats in the obese+irisin group (Table 1).

Uterine histopathology

As shown in Fig. 3, the uterine epithelium, uterine glands in the endometrium, and endometrial connective tissue fibers showed normal histological structure in the control, irisin, and obese+irisin groups. Unlike other groups, epithelial degeneration was determined to be more common in the uterine sections of high-fat diet-induced obese rats (Fig. 3).

Ovarian histopathology

Histological features of ovarian sections are shown in Fig. 4. Tissue sections from the ovary of vehicle-treated rats show common ovary histology with germinal epithelium consisting of single-layered cubic cells, numerous corpus luteum,

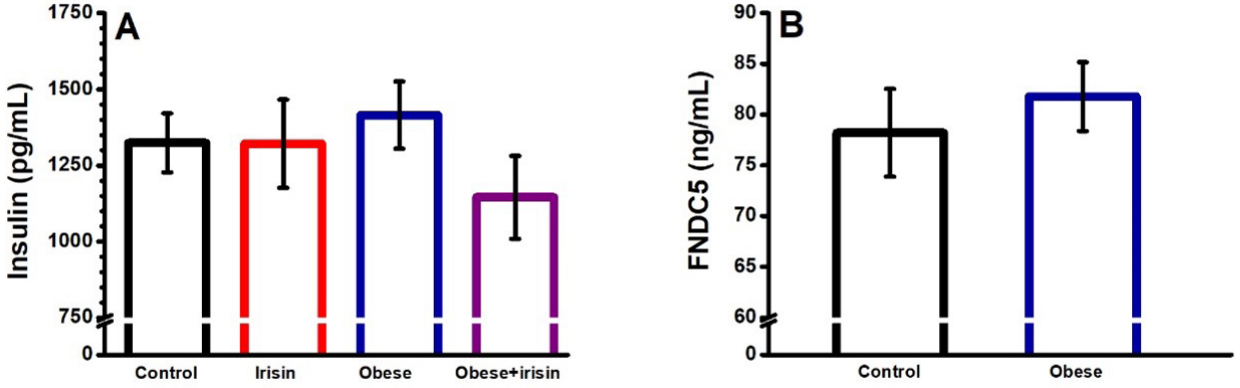

Figure 2: Effects of subcutaneous irisin perfusion and/or high-fat diet-induced obesity on A) serum insulin levels and B) serum FNDC5 levels in female rats. Data were expressed as mean ± SEM (one-way ANOVA followed by Tukey's *post-hoc* test or Student's t-test, n = 10 in each group)

Table 1: Effects of subcutaneous irisin perfusion and/or high-fat diet-induced obesity on reproductive organ weights in female rats. Data were expressed as mean ± SEM. *p<0.05, **p<0.01, and ***p<0.001 compared with the control group (one-way ANOVA followed by the Tukey's *post-hoc* test, n = 10 in each group). BW: body weight

Groups	Normalized Ovarian Weight (mg/100 g BW)	Normalized Uterine Weight (mg/100 g BW)
Control	46.01 ± 2.39	165.21 ± 8.61
Irisin	48.47 ± 1.87	132.18 ± 8.38*
Obese	36.25 ± 1.94*	118.89 ± 8.75**
Obese+irisin	41.18 ± 2.09	113.22 ± 5.96***

Table 2: Effects of subcutaneous irisin perfusion and/or high-fat diet-induced obesity on the number of ovarian follicles and the histopathological score of fibrosis in female rats. Data were expressed as mean ± SEM. *p<0.05 compared with the control group (one-way ANOVA followed by the Tukey's *post-hoc* test, n = 6 in each group)

Variable	Control	Irisin	Obese	Obese+irisin
Primordial follicle	9 ± 1.22	8.6 ± 0.74	6.2 ± 1.06	8 ± 0.44
Primary follicle	10 ± 1	7.8 ± 1.77	6.6 ± 1.6	9.6 ± 0.97
Secondary follicle	9.6 ± 1.43	10.2 ± 1.15	8.6 ± 0.5	8.6 ± 0.67
Tertiary follicle	1.6 ± 0.4	1.6 ± 0.4	0.6 ± 0.24	1.6 ± 0.24
Corpus luteum	10.8 ± 1.31	12.2 ± 1.31	8.6 ± 1.32	9.4 ± 0.5
Fibrosis	0.33 ± 0.21	0.66 ± 0.33	1.83 ± 0.40*	1 ± 0.25

and ovarian follicles (primordial, primary, secondary, and tertiary follicles) during different stages of development in the cortex. Similar to the control group, many corpus luteum and ovarian follicles at different developmental stages were detected in the irisin, obese, and obese+irisin groups (Fig. 4 and Table 2). However, unlike the control group, extreme vascular dilatation and congestion in the ovarian cortical stroma and ovarian germinal epithelium degeneration were observed in the high-fat diet-induced obese rats (Fig. 4). Along with all these histopathological changes in the obese group, a significant increase in fibrosis was also found in the obese group compared to the control group ($p<0.05$, Table 2). On the other hand, when compared to the obese group, a decrease was observed in vascular congestion and germinal epithelial degeneration in the obese+irisin group depending on irisin exposure (Fig. 4).

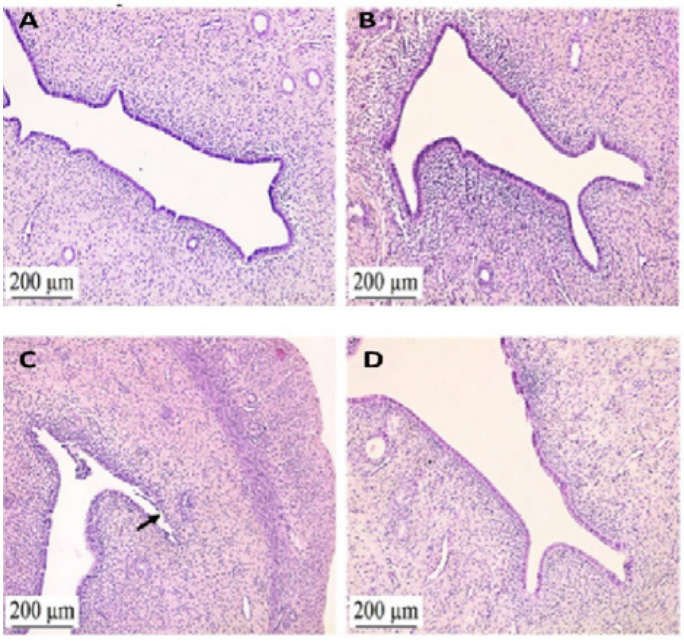

Figure 3: Represents the effects of subcutaneous irisin perfusion and/or high-fat diet-induced obesity on uterine tissue. A) Control group. B) Irisin group. C) Obese group: epithelial degeneration (arrow). D) Obese+irisin group. Stained with hematoxylin and eosin (H&E), Bar: 200 µm

Apoptosis in the ovary

Fig. 5A shows that TUNEL-positive granulosa cells were observed in the secondary and graff follicles, while apoptosis was not detected in the primordial and primary follicles in all experimental groups. Accordingly, we found that the apoptotic index (%) was significantly higher in the irisin, obese, and obese+irisin groups compared with the control group (control group: 1.71 ± 0.28%, irisin group: 5.42 ± 0.57%, obese group: 5.42 ± 0.61%, and obese+irisin group: 5.14 ± 0.59%; $p<0.05$ for irisin, obese, and obese+irisin groups, Fig. 5B).

Discussion

IIn this study, it was revealed for the first time that irisin hormone affects reproductive hormones and ovarian histopathology in high-fat diet-induced obese female rats. Accordingly, it was observed that irisin exposure increased serum LH, LH/FSH ratio, and progesterone levels in obese female rats, and the histopathological changes in the ovarian tissues of obese rats decreased with irisin exposure (i.e., vascular congestion and germinal epithelial degeneration).

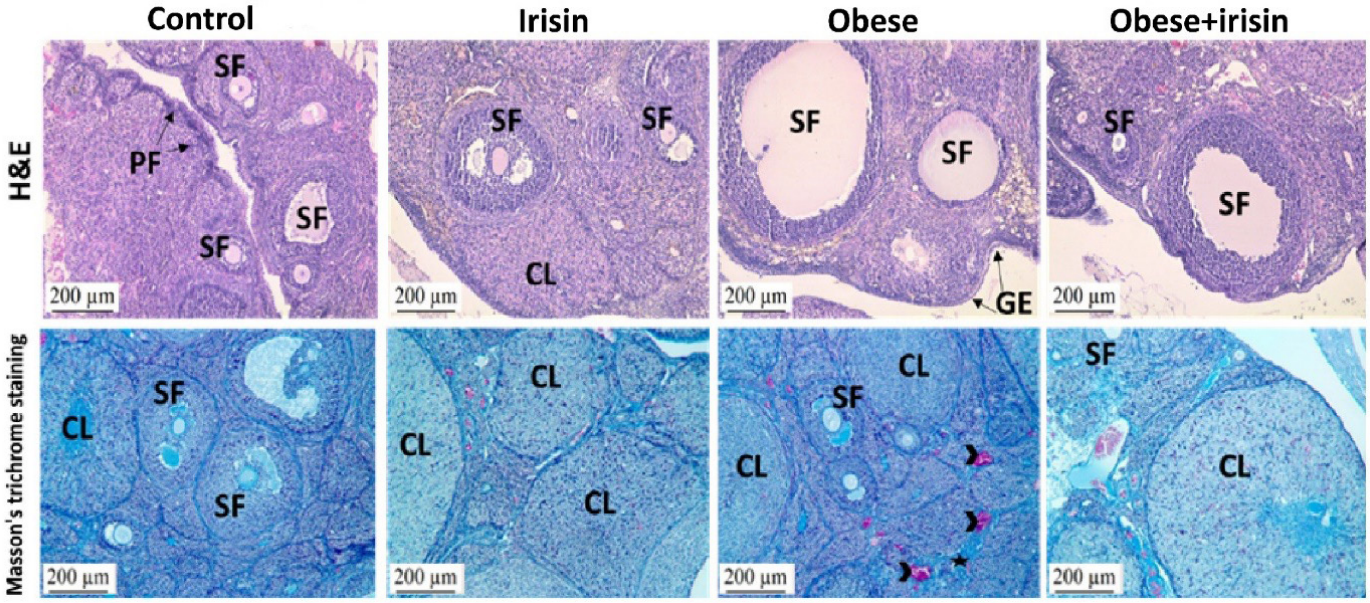

Figure 4: Represents the effects of subcutaneous irisin perfusion and/or high-fat diet-induced obesity on ovary histology. Obese group: vascular dilatation and congestion (arrowhead), fibrosis (star), and germinal epithelial degeneration (arrow). Stained with hematoxylin and eosin (H&E) and Masson trichrome staining, Bar: 200 µm. CL: corpus luteum; GE: germinal epithelium; PF: primordial follicle (in the control group, arrow); SF: secondary follicle

In addition, it was determined that endogenous irisin serum concentration did not change in the obese female rats, and exogenous irisin administration induced apoptosis in the lean rat ovary.

It has been shown in the literature that irisin/FNDC5 is most likely involved in the reproductive system. In female mice, it has been determined that there are various disturbances in the components of the female reproductive system due to the deletion of FNDC5. In some studies conducted in rat models, it was suggested that irisin had a beneficial effect on uterine receptivity or caused disorders in the reproductive system (9, 12, 22). Recent studies have shown that irisin has a potentially positive role against obesity-induced reproductive dysfunctions in male rats (15, 16).

The 'obesity epidemic' in many countries is a serious threat to public health, and reproduction is one of the major health hazards induced by obesity (23). It has been reported that obese women may have three times more reproductive disorders due to disruptions in the HPO axis, resulting in anovulatory cycles, irregular menstruation, and infertility, which are associated with PCOS (24, 25). In previous studies, it has been observed that anterior pituitary gonadotropins (FSH and LH) levels, which are critical regulators of ovarian function and female fertility, can increase in obese female rats. For example, Akamine et al. (2010) found that serum FSH levels did not change but serum LH levels were increased in obese female rats subjected to 120 days of high-fat diet treatment (26). Similar results on the secretion of gonadotropins were also found in female rats exposed to a 16-week high-fat diet in our study. Taken together, our data strongly suggest that obesity increases serum LH levels in female rats and thus causes an adverse effect on the gonadal axis of female rats.

Irisin either stimulates the expression of FSH and LH or inhibits the secretion of FSH or LH by competing with the gonadotropin-releasing hormone. These dual effects of irisin occur simultaneously and interact with each other, resulting in variations in circulating hormone levels when one activity is dominant (10, 12, 27). In the current study, it was determined that irisin decreased FSH levels in lean female rats by acting at the central inhibitory effect. In our previous study using female rats, it was determined that irisin administration changed serum levels of gonadotropins, resulting in reduced serum FSH levels and elevated serum LH levels (12). Similarly, in the present study, irisin was found to decrease serum FSH levels. However, the unchanged LH levels in the present study are thought to may be related to the application time and method of the irisin (i.e., 4 weeks versus 10 weeks and subcutaneous versus intraperitoneal administration).

On the other hand, when the effects of irisin on the secretion of gonadotropins, which changed due to obesity, were examined, it was shown that FSH levels did not change but LH levels and the LH/FSH ratio increased by irisin exposure in high-fat diet-induced obese female rats in this study. These results are supported by a study of the exogenous administration of irisin in obese female mice induced by a high-fat diet (13). Another possible condition in which LH levels and the LH/FSH ratio can increase in obese women is known to be PCOS (28, 29). Because of this, depending on the results of this study, it is speculated that irisin may have a triggering effect on the formation of PCOS in obesity.

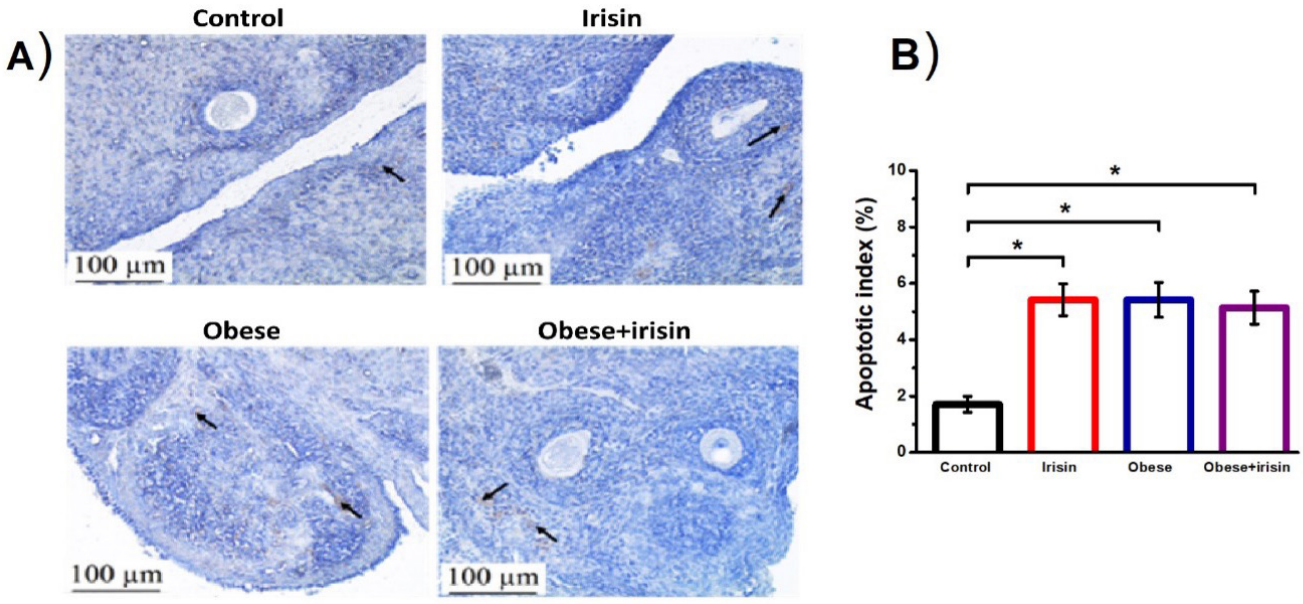

Figure 5: Effects of subcutaneous irisin perfusion and/or high-fat diet-induced obesity on the apoptosis of ovarian granulosa cells in rats. A) Representative images of TUNEL staining show apoptotic granulosa cells in the control, irisin, obese, and obese+irisin groups (arrow), Bar: 100 µm. B) The apoptotic index of each group. Data were expressed as mean ± SEM. *p<0.05, compared with the control group (one-way ANOVA followed by the Tukey's *post-hoc* test, n = 6 in each group)

Serum leptin levels are high in obesity, and an increase in leptin levels impairs ovulation and causes infertility. Moreover, it has been reported that serum leptin levels are negatively correlated with serum estradiol levels in obese female rats, and increased serum leptin levels may reduce estradiol synthesis by direct action on the ovary (30). Our previous study demonstrated that serum leptin levels were increased in obese female rats, and irisin administration decreased the elevated serum leptin levels in these rats (18). Therefore, in the present study, although serum leptin levels were not measured, it is speculated that the possible obesity-related increase in serum leptin levels may affect the ovary in obese rats and cause a decrease in E2 synthesis. A previous study reported that serum estradiol concentration decreased and serum progesterone concentration did not change in cafeteria diet-induced obese female rats (24). Consistently, similar results were also found in obese female rats induced by a high-fat diet in the present study.

On the other hand, it was determined that subcutaneous irisin perfusion increased serum progesterone levels in both lean and obese rats. In addition to the increase in progesterone levels, it was determined that there was also an increase in serum testosterone levels in the obese+irisin group. It is already known that LH is the major factor in ovarian steroidogenesis and triggers steroid production, including estradiol, progesterone, and testosterone (31). Therefore, considering the increasing effect of irisin on the LH levels in obese female rats as stated above, our data suggest that irisin-induced LH increment, independent of insulin, may increase steroidogenic capacity in the obese+irisin group.

In our study, serum insulin levels were unchanged in female rats fed a high-fat diet for 16 weeks compared to vehicle-treated female rats. Parallel to our study, Lu et al. (2016) reported that serum insulin levels did not change in Wistar male rats fed a high-fat diet for 24 weeks (32). Insulin plays an important role in the increase in ovarian steroidogenesis and the process of follicular development (26, 33). Especially, it has been revealed that there is an increase in theca-cell steroidogenesis as a result of the synergistic interaction of LH and insulin (34). In the present study, it was assumed that the changes in ovarian steroidogenesis in all experimental groups (i.e., increased progesterone levels in the irisin group, decreased E2 levels in the obese group, and increased progesterone and testosterone levels in the obese+irisin group) may have occurred independently of insulin. Furthermore, it is the first time, to our knowledge, that obesity-related reproductive dysfunction in a female rat model was found to be independent of serum irisin levels, considering the unchanged serum irisin levels in obesity in the current study.

Different exercise intensities, known to elicit an irisin response, induce different effects on the reproductive system in females. For example, it was observed that there is no change in serum FSH, LH, or estradiol levels as a result of short-time exercise or aerobic exercise in young women (35, 36), while there is a decline in the plasma levels of FSH, LH, estradiol, and progesterone in women who engage in regular high intensity exercise (37). Depending on the results of the present study and our previous study (12), it was determined that irisin administration at different durations and ways has different effects on reproductive hormones as well as ovarian histology and reproductive organ weights in female rats. In light of the above-mentioned information, our data from our studies suggested that the irisin hormone released into circulation due to exercise may play a pivotal role in the relationship between exercise variables and the reproductive system.

Gaspar et al. (2016) and Benevides et al. (2019) reported that both the weights of the uterus and ovarian tissues were reduced due to obesity in rats (38, 39). Hence, the marked reduction in reproductive organ weights seen in high-fat diet-induced female rats in the present study is consistent with previous literature and provides evidence of the direct adverse effects of obesity on reproductive organs. Besides, for the first time in the current study, it was revealed that irisin did not affect the weight of reproductive organs in obese female rats.

In rodent models, it is also known that obesity causes different histopathological changes in ovarian tissue as well as negative effects on the weight of reproductive organs. Akamine et al. (2010) showed that rats fed a high-fat diet for 180 days had significantly abnormal ovarian morphology (26). In addition, Atteia et al. (2020) revealed that there are several histopathological features such as stromal edema, congestion, and a high degree of fibrosis in the ovarian cortices of the obese group (40). Our findings agree with the aforementioned studies: obese rats showed histopathological changes in the ovaries like extreme vascular dilatation and congestion in the ovarian cortical stroma, ovarian germinal epithelium degeneration, and fibrosis. With regards to the ovarian follicle reserve, obesity did not affect ovarian follicle development in the current study. Benevides et al. (2019) and Hussain et al. (2016) found similar results: obesity did not promote a significant change in the count of ovarian follicles or corpora lutea (39, 41). In particular, similar follicular development in all experimental groups is an expected result due to the unchanged serum levels of insulin involved in follicular development in this study.

It is noteworthy that irisin exposure reduced the changes in ovarian pathology (i.e., vascular congestion and germinal epithelial degeneration) in obese rats, while it did not cause any effect on the ovarian tissues of lean rats in the present study. Accordingly, in this study, it could be speculated that irisin exhibited a curative effect on obesity-induced pathological changes in the ovarian tissues. Taken together, our data suggested that irisin can act directly on the ovaries in obese rats, regardless of the HPO axis.

In the present study, the effects of irisin on ovarian function in rats fed a high-fat diet were also assessed by apoptosis

in addition to hormone assays and histopathological examinations. Our data showed that apoptosis in the ovary was increased, which contributes to ovarian function failure, by irisin exposure alone or obesity alone in rats, but the administration of irisin did not affect granulosa cell apoptosis in obese rats. This effect of obesity on apoptosis of ovarian follicles shown in this study is supported by recent studies demonstrating that diet-induced obesity is known to increase apoptotic ovarian follicles in rodents (13, 24, 42). Irisin has also been demonstrated to increase apoptosis in ovarian cancer cells and breast cancer cells (43, 44). Similarly, to our knowledge, our findings have shown for the first time that irisin induces apoptosis in the ovarian tissue of lean rats. In conclusion, we have demonstrated that reproductive impairments in irisin-exposure rats in the present study may be associated with decreased serum FSH levels, increased serum progesterone levels, decreased uterine weights, and increased granulosa cell apoptosis.

In conclusion, our study revealed that irisin increased serum LH levels in high-fat diet-induced obese female rats, and irisin-induced LH increment may cause an increase in ovarian steroidogenesis in these obese rats. In addition, it was shown that irisin exposure could alleviate ovarian tissue damage in high-fat diet-induced obese rats. Based on these results, we suggest that the irisin hormone may modulate the HPO axis of obese rats at both central neuroendocrine and gonadal levels.

Conclusions

It was determined that λ-cyhalothrin has a toxic effect on *D. polymorpha* and this toxic effect increases depending on the temperature. In our study, it was concluded that the *D. polymorpha* model organism and some of its biochemical parameters (AChE, CAT, SOD, GSH and MDA) are suitable biomarkers for revealing the effect of temperature variable on toxic response. It has also been shown that biochemical parameters such as SOD, CAT, AChE activities and GSH, TBARS levels used are suitable biomarkers for the evaluation of the toxic effects of λ-cyhalothrin.

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Author contributions: SC and HK conceived and designed the project. NUE, AY, and FB performed all animal experiments. NUE performed all hormone measurements and wrote the manuscript. NKT carried out all histological studies and the TUNEL assay. SC and MO analyzed and interpreted the data. The final version of the manuscript was read and approved by all authors.

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Učinki irisina na reproduktivni sistem debelih samic podgan, povzročeni s prehrano z visoko vsebnostjo maščob

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Izvleček: Debelost je vse pogostejša po vsem svetu in povzroča različne zdravstvene težave, vključno z motnjami reprodukcije. Čeprav hormon irisin, ki se izloča med vadbo, lahko vpliva na hipotalamično-hipofizno-gonadno os in reproduktivno funkcijo, njegov vpliv na z debelostjo povezane poškodbe ženskega reproduktivnega sistema ni povsem znan. Zato je bil namen te študije raziskati morebitne učinke irisina na reproduktivne hormone in reproduktivne organe pri samicah podgan z debelostjo, povzročeno s prehrano z visoko vsebnostjo maščob. Štirideset samic podgan smo razdelili v štiri skupine: kontrola, irisin, debelost, debelost+irisin (n=10 v vsaki skupini). Po 12 tednih simulacije modela debelosti, povzročene s prehrano z visoko vsebnostjo maščob (60 % kcal maščobe), smo v skupinah debelost in debelostjo+irisin podganam podkožno dajali irisin (100 ng/kg/dan prek mini-osmotskih črpalk približno 28 dni). Podkožna aplikacija irisina je povečala serumski luteinizirajoči hormon (LH), razmerje med LH in folikle stimulirajočim hormonom (FSH) (LH/FSH) in raven progesterona, hkrati pa zmanjšala histopatološke poškodbe v jajčnikih debelih podgan. Vendar pa so bile koncentracije endogenega irisina v serumu vitkih in debelih podgan z reproduktivnimi motnjami podobne. Rezultati kažejo, da bi irisin lahko vplival na reproduktivno os debelih podgan. K temu učinku bi lahko prispevala povečanje serumske koncentracije LH, kar sproža steroidogenezo jajčnikov, ter zmanjšanje histopatoloških sprememb tkiva jajčnikov.

Ključne besede: : irisin; debelost; hormoni; jajčnik; apoptoza; reprodukcija pri samicah

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Original Research Article

Prioritization of Candidate Genes for the Effect of Fob3b1 QTL on Chromosome 15 in Mouse Models for Polygenic Obesity and Leanness using Integrative Genomics

Key words

data integration;
gene expression;
gene prioritisation;
mouse models;
obesity;
QTL;
single nucleotide polymorphism

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Abstract: The accumulation of excess fat affects meat quality, fertility, productivity, and whole-body metabolism in farm animals. The mouse model presents an efficient tool for investigating these traits. Previous QTL analyses of the unique mouse selection lines for polygenic obesity (Fat line) and leanness (Lean line) have revealed four major obesity QTLs: *Fob1*, *Fob2*, *Fob3*, and *Fob4*. *Fob3*, located on chromosome 15, was later subdivided into *Fob3a* and *Fob3b*, which additionally split into *Fob3b1* and *Fob3b2*. Of the 158 genes annotated in *Fob3b1*, 16 candidate genes have been previously proposed for the QTL effects. However, genomic variability between the Fat and Lean lines at this locus has not been fully investigated. The present study aimed to validate previously identified candidates and to identify novel candidate genes potentially responsible for the *Fob3b1* effect. Data from whole-genome sequencing and transcriptome analyses of Fat and Lean mouse lines were integrated with obesity QTLs in cattle and pigs from Animal QTLdb and phenotypes obtained from the International Mouse Phenotyping Consortium (IMPC) and the Mouse Genome Database (MGD). Out of 158 genes located in the *Fob3b1* interval we prioritized 17 candidate genes, including six previously proposed (*Adgrb1*, *Col22a1*, *Cyp11b1*, *Dgat1*, *Gpihbp1* and *Ly6a*) and 11 novel candidates: *9030619P08Rik*, *Eppk1*, *Kcnk9*, *Ly6c1*, *Ly6d*, *Ly6h*, *Ly6i*, *Ly6m*, *Ptk2*, *Trappc9*, and a strong candidate Ly6e that deserve further functional analyses. Biological function and literature screening for candidate genes suggest that the *Fob3b1's* impact on obesity may operate through triglyceride metabolism (*Dgat1* and *Gpihbp1*) and cytoskeletal and extracellular matrix remodelling (*Ly6a*, *Ly6e* and *Eppk1*). Further fine mapping, genetic and "omic" studies should clarify whether the *Fob3b1* effect is due to a causal genetic variant in one of the candidates or possibly due to an additive effect of a combination of these positional candidates. The applied bioinformatics approach in determining the priority of candidate genes for obesity can also serve as a model for other traits in veterinary and livestock sciences.

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Introduction

Obesity, considered by many to be the epidemic of the 21st century, is broadly divided into two categories: the monogenic type and the more common polygenic type (1,2). Obesity leads to the development of metabolic disorders such as diabetes mellitus, high blood pressure, cardiovascular diseases, and inflammation-related diseases (3). The accumulation of excess fat also affects meat quality, fertility, productivity, and whole-body metabolism in farm animals

and is also one of the most important health and welfare issues affecting companion animals (4). Rodent models such as mice and rats serve as invaluable tools for studying the complex biology of obesity, identifying new therapeutic targets, and evaluating the efficacy and safety of potential interventions (5). There are few mouse models for the polygenic type of obesity, but they have no lean counterparts derived from the same base population. Selective breeding for desired divergent phenotypes over an extended period creates novel, polygenic, and reproducible disease models (6,7). One such mouse model was developed by divergent selection on body fat percentage over more than 60 generations, resulting in the Fat and Lean lines, which differ in fatness by a factor of five (8).

Earlier genome-wide quantitative trait locus (QTL) analyses of the two selection lines revealed four major obesity QTLs (*Fob1, Fob2, Fob3*, and *Fob4*) (9). Further experimental data showed that the QTL interval with the highest LOD (logarithm of the odds) score, Fob3 on chromosome 15, consists of two linked QTLs with smaller effects, *Fob3a* and *Fob3b* (10), which additionally split into *Fob3b1*, with a stronger effect, and *Fob3b2* (11). Sixteen candidate genes have been proposed for the *Fob3b1* effect based on previously identified obesity QTLs in mice and cattle, gene expression analyses obtained from the expression database, and based on their known biological functions (11). However, the genomic variability and differential gene expression between the Fat and Lean lines at this locus, which could significantly improve the prioritisation power for candidate genes, have not yet been fully investigated.

In the present study, integration of whole genome sequencing (WGS) focusing on single nucleotide polymorphisms (SNPs) and gene expression data of genes within *Fob3b1* in white adipose tissue of the Fat and Lean lines were performed to prioritise candidate genes responsible for the *Fob3b1* effect. In addition, candidates were complemented with their relatedness to obesity using gene and gene knock-out annotations and a comparative genomics approach between mouse, pigs, and cattle.

Material and methods

Whole genome sequencing (WGS) data of Fat and Lean mice were from our previous studies (12) (13). Differential gene expression data for three white adipose tissues (WAT) depots (epididymal WAT, subcutaneous WAT, mesenteric WAT) were from (14). The expression data from the three tissues were then joined and corrected for the batch effect using Empirical Bayes Analysis to obtain expression data in WAT. Gene expression was considered differential if expression differed between Fat and Lean mouse lines by at least 1.5-fold. Significance was checked at both $p < 0.05$ and adjusted $p < 0.05$ (differentially expressed genes; DEGs).

Two criteria were used for the candidate gene prioritisation for the *Fob3b1* effect: 1.) genes carried line-specific SNPs within coding regions (exons) according to the Ensembl Variant Effect Predictor (https://www.ensembl.org/Tools/VEP) (15) or 2.) genes were differentially expressed in WAT between the Fat and Lean mouse lines. The results were complemented with annotations related to obesity by the International Mouse Phenotyping Consortium (IMPC, www.mousephenotype.org) (16) using the search term "abnormal adipose tissue amount" and the Mouse Genome Database (MGD; http://www.informatics.jax.org) (17) using the search term "fat" in the terms for mammalian phenotypes. In addition, previous gene associations with obesity were extracted by literature screening using the Pubmed database and MeSH Terms adipog*, obes*, fat, lipid droplet and approved gene symbols and synonyms. The candidate genes were supplemented with orthologous genes within obesity-related QTLs in cattle and pigs, obtained using Animal QTLdb (https://www.animalgenome.org/cgi-bin/QTLdb/, Release 50, April 25, 2023) (18), Ensembl (19), and g:Profiler (20). First, the locations of QTLs related to subcutaneous fat/adipose thickness/amount were obtained from Animal QTLdb. Second, genes within these QTLs were identified using the Ensembl Biomart. Finally, orthologous genes in mice were obtained from g:Profiler. The genomic location of *Fob31b* was obtained by converting genomic coordinates provided by (11) (71.38– 76.36 Mbp, mouse NCBI36 assembly) to genome assembly GRCm38 using UCSC Genome Browser tool liftOver (https://genome.ucsc.edu/cgi-bin/hgLiftOver). Location of regulatory elements (open chromatin, enhancer, promoter, promoter flanking region, or CTCF binding site) was obtained from Ensembl database.

Results

The present study aimed to prioritize genes responsible for the *Fob3b1* effect in mouse models for polygenic obesity and leanness. Two criteria were used for the candidate gene prioritisation: 1.) line-specific SNPs in coding regions or 2.) differential gene expression in WAT between the Fat and Lean mouse lines. The workflow with the main results is shown in Figure 1.

The *Fob3b1* interval, spanning from 15:71,550,331-76,532,745, contains 158 genes (GRCm38) of which 67 genes carry line-specific SNPs, and seven were differentially expressed in WAT between the Fat and Lean lines. By prioritization of 158 genes, we obtained 17 promising candidates: 10 genes with SNPs in coding regions (seven genes with missense variants, three genes with synonymous variants), six genes with differential expression, and *Ly6e* with both synonymous exonic variants in the Lean line and differential expression (Table 1). We were also interested if differential expression might be caused by potential regulatory variants (SNPs located within open chromatin, enhancer, promoter, promoter flanking region, or CTCF

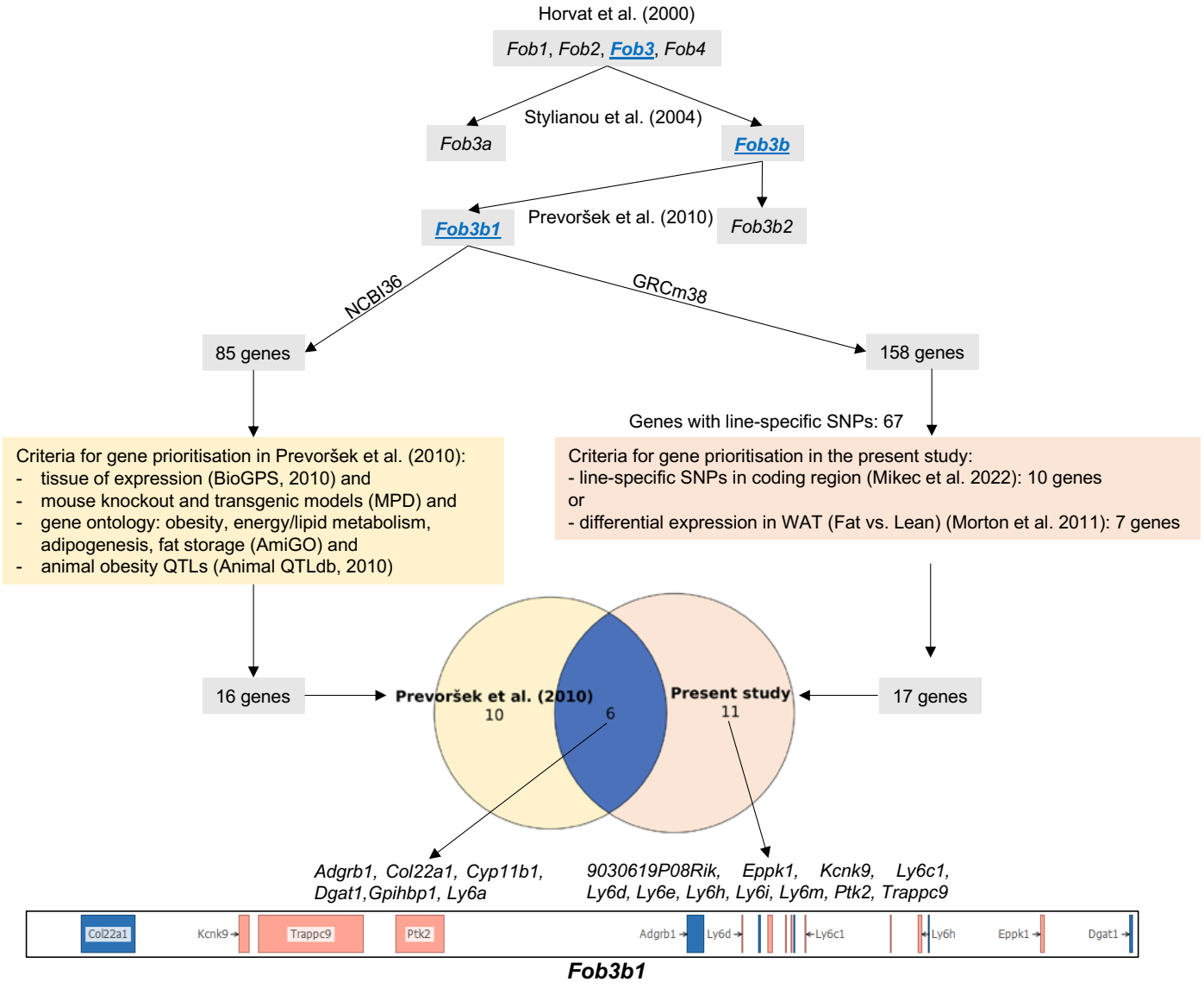

Figure 1: The workflow of the study for the prioritization of candidate genes responsible for the Fob3b1 effect

binding site). Among the DEGs, two genes, both in the Lean line, carry potentially regulatory variants that may affect their expression. In *9030619P08Rik*, the SNP rs31762288 is located within the open chromatin region and rs13482652 and rs32099107 are within promoter flanking region. As for *Ly6e*, all 42 potentially regulatory variants are within promoter flanking region. Out of 17 QTL prioritized candidates six were proposed previously (*Adgrb1*, *Col22a1*, *Cyp11b1*, *Dgat1*, *Gpihbp1*, and *Ly6a*) (11), while the *9030619P08Rik*, *Eppk1*, *Kcnk9*, *Ly6c1*, *Ly6d*, *Ly6e Ly6i*, *Ly6h*, *Ly6m*, *Ptk2*, and *Trappc9* are newly proposed candidate genes. Some of the 17 candidate genes have been previously associated with obesity, but genes, such as *Col22a1*, *Eppk1*, *Ly6i*, and *Ly6m* have been proposed to be associated with obesity for the first time. The comparative genomics approach revealed 14 orthologous genes located within the obesity-related QTLs in cattle, however, none of them is located within the obesity QTLs in pigs (Table 1).

Discussion

In the present investigation, we undertook a comprehensive analysis by integrating whole genome sequencing (WGS) and transcriptomics data from the Fat and Lean mouse lines to systematically prioritize candidate genes responsible for the observed effects of *Fob3b1*. Our specific emphasis was directed toward SNPs in coding regions and the gene expression profiles of genes within the *Fob3b1* locus in WAT. For the SNPs in coding regions, we also included synonymous variants as accumulating experimental evidence has demonstrated that they may exert their impact on gene functions via splicing accuracy, mRNA stability, translation fidelity, protein folding, and expression (21).

As many as seven out of the candidate genes in the present study are part of the LY6 (lymphocyte antigen 6 complex) family of proteins involved in a variety of functions in cell proliferation, migration, cell-cell interaction, immune cell

Table 1: 27 positional candidate genes for *Fob3b1* effect; 16 from the study Prevoršek et al. (2010) (8), 17 from the present study (marked in bold), including six genes identified by both studies

	Priority	Gene	SNPs[1]	Criteria 1: Line specific SNPs in coding region	SNP located within regulatory region	Criteria 2: DEG2	IMPC[3]	MGI[3]	Cattle QTL[4]	Literature associated with obesity
Prevoršek et al. (2010)	high	***Dgat1***	B:17		B:2	↑		√		√
		Gpihbp1	/			↑			√	√
		Rhpn1	B:1						√	
		Ly6a	L:56	L:3	L:9			√		√
	moderate	***Cyp11b1***	L:27, F:1	L:1				√	√	√
		Cyp11b2	L:47		L:2				√	√
		Gpr20	/						√	√
		Adgrb1	L:1, F:1	L:1	L:1				√	
		Tsta3	/							
	low	*Arc*	/						√	√
		Psca	/						√	
		Ly6g2	L:104		L:13					
		Gsdmd	/						√	√
		Naprt1	/						√	√
		Cyc1	B:1							√
		Col22a1	L:388, F:499, B:283	L:2, F:8, B:4	L:17, F:21, B:12					
Present study		***Ly6e***	L:106	L:2	L:42	↑			√	
		Trappc9	L:1144, F:3, B:3	L:8	L:197, B:2		√	√	√	√
		9030619P08Rik	L:22		L:3	↑				
		Ly6d	/			↑			√	
		Ly6h	/			↑			√	
		Eppk1	/			↑				
		Kcnk9	L:13	L:1	L:4					√
		Ly6c1	L:49	L:1						√
		Ly6i	L:148	L:3	L:7					
		Ptk2	F:2	F:1	F:1					√
		Ly6m	L:65, B:2	L:3	L:7					

[1]SNPs identified in both (B), Fat (F) or Lean (L) lines, [2]Differentially expressed gene (Fat vs. Lean); ↑: upregulated, [3]Associated with obesity-related traits in IMPC and MGI databases, [4]Orthologous genes in obesity-related QTL in cattle obtained from Animal QTLdb

maturation, macrophage activation, and cytokine production, mainly by regulating acetylcholine signalling (22) that has been recently linked to insulin sensitivity, low-grade inflammation, adipose dysfunction and metabolic syndrome in obesity (23,24). While four of them (*Ly6a, Ly6c1, Ly6i, Ly6m*) carry exonic variants in the Lean line, the remaining three (*Ly6d, Ly6e*, and *Ly6h*) were found to be expressed to a higher level in WAT of the Fat line compared to the Lean line. In addition, higher expression of an uncharacterized *9030619P08Rik*, described as an LY6 pseudogene (25), and *Gpihbp1*, a member of the LY6 superfamily (26), was determined in the Fat line WAT. The expression of *Ly6d*, *Ly6h*, and *Gpihbp1* did not depend on regulatory SNPs in our study, suggesting that there may be genetic variations in the transcriptional regulators of these three genes located elsewhere in the genome. Meanwhile, *Ly6e* and *9030619P08Rik* in the Lean line carry potential regulatory variants that may explain their higher expression levels in the Fat line.

Among these genes, *Ly6ci*, *Ly6a*, and *Ly6e* are especially worth mentioning. While *Ly6ci* was linked to abnormal metabolic pathways in the early induction phase of autoimmune diabetes (27), altering T cell function (28), *LY6A* and *LY6E* were, in addition to their involvement in immunity (29,30) also linked to extracellular matrix remodelling (31,32). In adipose tissue of obese individuals, remodelling of the extracellular matrix, cytoskeletal reorganisation and increased cell proliferation enable the enlargement of obese adipocytes and WAT expansion (33,34). The *Ly6a* is not differentially expressed, however, only the Lean line carries SNPs, including two missense variants rs213983347 (V/A) and rs32279213 (D/G), located in the same exon and within the protein domain Ly-6 antigen/uPA receptor-like, suggesting their effect on protein function. In cattle, *LY6A* has been associated with fertility, potentially by affecting growth dynamics in the unborn calf (35), and *LY6D* is crucial for lipid accumulation and inflammation in nonalcoholic fatty liver disease (36). Meanwhile, *Eppk1*, a new candidate gene with higher expression in the Fat line, is involved in cytoskeleton reorganization and cell proliferation (37). *Col22a1*, which encodes an extracellular matrix protein, is not differentially expressed but has several exonic variants in the Lean and Fat lines. *COL22A1* has been shown to increase intramuscular fat in cattle (38) and polymorphisms in porcine *COL22A1* were associated with daily weight gain (39).

Moreover, an uncharacterized *9030619P08Rik* is thought to be translated into a stable circulating microprotein that may be involved in metabolic regulation and obesity (25), and *Gpihbp1* regulates the lipolytic processing of triglyceride-rich lipoproteins (26). Nucleotide substitutions in *GPIHBP1* cause lifelong chylomicronemia (40). Lipolysis of triglyceride-rich particles leads to lower protective HDL cholesterol levels (41), which was previously observed in the Fat compared to the Lean line (42). Furthermore, changes in (high basal/low stimulated) lipolysis rates are associated with insulin resistance, previously demonstrated in the Fat line (43), and future weight gain in humans (44). Some polymorphisms in porcine *GPIHBP1* were proposed to be genetic risk factors affecting adipose traits (45).

Among the high-priority candidates from a previous study (11) the expression of *Gpihbp1* and *Dgat1* was found to be higher in WAT of the Fat line, although the sequences in the two lines were identical. *DGAT1* catalyses the final step of triglyceride synthesis (46), and *Dgat1*-deficient mice are lean and resistant to diet-induced obesity (47). In addition, DGAT1 was associated with a backfat thickness (48), fat deposition (49), and intra-muscular fat in pigs (50), and beef marbling (51). It was also identified as one of very few causative genes for milk yield and composition - fat content in cattle (52)

Other potential candidates include *Cyp11b1*, *Adgrb1*, *Kcnk9*, *Trappc9*, and *Ptk2*. Twenty-six of 27 line-specific SNPs were identified in the Lean line *Cyp11b1*, including a synonymous rs31832746. CYP11B1 is a rate-limiting enzyme in the synthesis of cortisol (53), an obesity-related steroid hormone (54) whose formation selectively increases within adipose tissue in obesity (55). Even more promising candidate for QTL effect is *Adgrb1*, with a potentially deleterious variant rs51566550 in the Lean line. *Adgrb1*$^{-/-}$ mice exhibited increased susceptibility to seizures, delayed growth, and reduced brain weight (56). ADGRB1 is involved in a membrane-initiated pathway to induce the expression of *Abca1* (ATP-binding cassette, sub-family A (ABC1), member 1) in apoptotic cells (57) whose specific knockout in adipocytes resulted in significantly lower body weight, epididymal fat pad weight and adipocyte size due to changes in lipogenesis and lipid accretion in mice (58). Additionally, it is noteworthy that ADGRB1 may play a role in sensory food perception (59), which alone can cause metabolic changes (60,61). Similarly, *Kcnk9* encodes TWIK-related acid-sensitive K channel 3 (TASK3) protein that has been implicated in glucose sensing (62). *Kcnk9* transcript was significantly up-regulated in mice nodose neurons fed a high-fat diet. The authors proposed it as a therapeutic target for obesity treatment (63). Adipose-specific knockout of a closely related gene *Kcnk3* in mice resulted in an increased energy expenditure and resistance to obesity (64). A SNP rs2471083 near the potassium channel *KCNK9* has a parent-of-origin effect on body mass index (65) and was linked to abdominal visceral fat by GWAS (66). Another candidate is *Trappc9*, with eight synonymous variants in *Trappc9* of the Lean line. This gene plays a role in energy balance, and its deficiency leads to obesity (67). It has been linked to fat deposition-related traits in Hu sheep (68) and to body size traits in pigs (69). PTK2 (also known as focal adhesion kinase FAK), best known for its involvement in integrin signalling, was shown to influence adipocyte differentiation and to influence obesity in mice (70). In addition to its role in leptin signal transduction (71), FAK signalling controls insulin sensitivity through the regulation of adipocyte survival (72), and FAK inhibition causes insulin resistance (73). A novel missense variant 15_73264244_G/T in the Fat line may therefore

Table 2: Candidate orthologous genes associated with milk traits in cattle and pigs.

Gene/Region	Effect on milk	Species/breed	Reference
ADGRB1	urea content	Holstein cattle	Ma et al. (2023) (79)
ADGRB1	lactose content	Fleckvieh cattle	Costa et al. (2019) (75)
	yield		
CYP11B1	yield	German Holstein cattle	Kaupe et al. (2007) (35)
DGAT1	protein content	Polish landrace pigs	Szyndler-Nędza and Piorkowska (2015) (74)
	lactose content		
	fat content	Romanian Holstein cattle	Tăbăran et al. (2015) (81)
GPIHBP1	fat content	cattle	Yang et al. (2017) (80)
	protein content		Dong et al. (2020) (76)
LY6E	yield	Holstein cattle	Jiang et al. (2018) (77)
TRAPPC9	protein content	Chinese Holstein cattle	Khan et al. (2022) (78)
	mastitis resistance		

influence various signalling pathways and contribute to the obese phenotype.

However, the prioritised candidate genes may play other roles in tissues not examined in the present study. Interestingly, *DGAT1*, *GPIHBP1*, *CYP11B1*, *ADGRB1*, *LY6E*, and *TRAPPC9* are also associated with milk production and milk composition traits in cattle and pigs, such as milk urea, lactose, protein, and fat contents and milk yield (35,74–81) (Table 2). Importantly, recent metabolomic and proteomic investigations revealed a correlation between infant obesity and milk composition from obese or non-obese mothers (82,83). Considering *Cyp11b1*, *Adgrb1*, *Ly6e*, and *Trappc9*, the Lean line carries exonic variants that may affect the protein function, these genes may also affect milk composition and yield and subsequently contribute to the lean or obese phenotype in our mouse models.

In summary, *Fob3b1* may influence energy balance, inflammation, various signalling pathways (acetylcholine, leptin, insulin), metabolism, and cell structure in WAT, however, it may also contribute to the obese/lean phenotype by influencing milk quantity and composition. For the *Fob3b1* effect on the adiposity of WAT, we propose genes involved in triglyceride metabolism (*Dgat1* and *Gpihbp1*), cytoskeleton, and extracellular matrix remodelling (*Ly6a*, *Ly6e*, and *Eppk1*) as the main contributors, calling for their future functional analyses.

The control of fat deposition, energy metabolism, and immune system functioning have high economic importance in farm animals. Excess fat accumulation affects meat quality, fertility, productivity, and whole-body metabolism (84). Further functional studies of the proposed candidate genes are required to elucidate their involvement in fat deposition.

Conclusions

The present study identified 17 candidate genes potentially responsible for the *Fob3b1* QTL effect in mouse models for polygenic obesity and leanness. In particular, triglyceride metabolism, cytoskeleton and extracellular matrix remodelling may be the main contributors to the effect of *Fob3b1*. Of the 17 most promising candidate genes, four new obesity candidates were proposed: *Col22a1*, *Eppk1*, *Ly6i*, and *Ly6m*. Further work on fine mapping and functional analyses is required to determine whether the effect of *Fob3b1* is due to a causal genetic variant in one of these candidates or a combined effect of several of these positional candidates. The applied bioinformatics approach for prioritization of candidate genes for polygenic obesity in the present study can also be used to analyze other traits in veterinary medicine and livestock science. Obesity and its associated diseases pose a significant health risk, affecting not only physical well-being, but also reproductive health and overall animal welfare. These effects extend beyond farm animals to include companion animals, highlighting the interconnectedness of veterinary and human medicine in addressing obesity-related health problems in all species to improve animal health and welfare.

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Conflict of interest statement. All authors declare that they have no competing interests.

Authors' contributions. MŠ: Formal analysis, writing - original draft preparation. NMM: writing – review & editing. SH and TK: conceptualization, writing – review & editing, supervision.

Ethics approval and consent to participate. The FLI (Fat) and FHI (Lean) selection lines have been maintained in our laboratory for more than 100 generations. All mice used in this study were maintained according to local ethical and EU regulatory guidelines under the Veterinary Administration of Republic of Slovenia permit No. U34401-23/2020/6.

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Določanje prioritetnih kandidatnih genov znotraj intervala Fob3b1 QTL na kromosomu 15 pri mišjih modelih za poligensko debelost in vitkost z uporabo integrativne genomike

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Izvleček: Kopičenje odvečne maščobe vpliva na kakovost mesa, plodnost, proizvodnost in presnovo pri rejnih živalih. Mišji modeli predstavljajo učinkovito orodje za raziskovanje genetske osnove teh lastnosti. Predhodne analize QTL-ov edinstvenih mišjih selekcijskih linij za poligensko debelost (debela linija) in vitkost (vitka linija) so razkrile štiri glavne QTL-e za debelost: Fob1, Fob2, Fob3 in Fob4. Fob3, ki se nahaja na kromosomu 15, je bil kasneje razdeljen na Fob3a in Fob3b, zadnji pa se dodatno razdeli na Fob3b1 in Fob3b2. Od 158 genov, anotiranih v Fob3b1, je bilo v prejšnjih študijah predlaganih 16 kandidatnih genov. Vendar pa genomska variabilnost med debelo in vitko linijo na tem lokusu ni bila v celoti raziskana. Namen te študije je bil potrditi predhodno identificirane kandidate in identificirati nove kandidatne gene, ki bi lahko bili odgovorni za učinek Fob3b1. Podatki iz celotnega genoma sekvenciranja in transkriptomskih analiz debelih in vitkih mišjih linij so bili vključeni v primerjalno analizo s QTL-i za debelost pri govedu in prašičih iz Animal QTLdb ter fenotipi, pridobljenimi iz Mednarodnega konzorcija za fenotipizacijo miši (IMPC) in podatkovne zbirke mišjega genoma (MGD). Izmed 158 genov, lociranih v Fob3b1, smo prednostno obravnavali 17 kandidatnih genov, vključno s šestimi predhodno predlaganimi (Adgrb1, Col22a1, Cyp11b1, Dgat1, Gpihbp1 in Ly6a) in 11 novimi kandidati: 9030619P08Rik, Eppk1, Kcnk9, Ly6c1, Ly6d, Ly6h, Ly6i, Ly6m, Ptk2, Trappc9 in Ly6e. Biološka funkcija in pregled literature za kandidatne gene nakazujeta, da lahko učinek Fob3b1 na debelost deluje preko metabolizma trigliceridov (Dgat1 in Gpihbp1) ter preoblikovanja citoskeleta in zunajceličnega matriksa (Ly6a, Ly6e in Eppk1). Nadaljnje natančno kartiranje, genetske in »omske« študije bodo pojasnili, ali je učinek Fob3b1 posledica vzročnega učinka ene same genetske različice ali morda aditivnega učinka kombinacije večjega števila teh pozicijskih kandidatov. Uporabljeni bioinformacijski pristop pri določanju prednostne liste kandidatnih genov za debelost lahko služi tudi kot model za preučevanje drugih lastnosti v veterinarskih in živinorejskih znanostih.

Ključne besede: povezovanje podatkov; izražanje genov; razvrstitev genov po pomembnosti; mišji modeli; debelost; QTL; posamezni nukleotid; polimorfizem

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Case Report

Secondary Small Intestinal Obstruction Associated With an Omental Adhesion After End-To-End Jejuno-Ileal Anastomosis in a Thoroughbred Horse: A Case Report

Key words

colic,
horse;
jejuno-ileal nastomosis;
omental adhesion;
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Abstract: A 19-month-old female thoroughbred horse presented with a history of acute abdominal pain. When the horse was four months of age, she underwent abdominal surgery and most of the strangulated jejunum and ileum were resected and anastomosed using an end-to-end technique. Subsequently, the horse was diagnosed with an ileal obstruction secondary to an adhesion of the greater omentum, which caused a mechanical obstruction of the lumen of the distal ileum. The ileum was released by transecting the adhesion and performing an omentectomy. After surgical intervention, the horse recovered quickly and was discharged fifteen days after surgery. This case report describes an ileal obstruction caused by an omental adhesion that formed after a jejunoileal anastomosis in a thoroughbred horse. The clinical, imaging, and surgical findings are described.

Introduction

Postoperative intra-abdominal adhesions (PIAs) are complications of abdominal surgery that challenge equine surgeons (1). Fibrinous and omental adhesions are consequences of peritoneal inflammation that may remain clinically silent or cause complications depending on the location and organization (1). Pathological adhesions causing postoperative abdominal pain and intestinal obstruction have been reported in 9% to 27% of horses, identified at postmortem examinations or during repeat laparotomy after abdominal surgery (2). Furthermore, strangulating lesions of the small intestine associated with the omental adhesions (OA) occur in 1% of horses undergoing exploratory laparotomy for abdominal pain (3). This report describes the clinical signs, diagnosis, surgical findings, treatment, and follow-up of small intestinal obstruction (SIO) caused by an OA after an end-to-end jejuno-ileal anastomosis in a horse.

Case Presentation

A 19-month-old female thoroughbred horse, weighing 430 kg presented with anorexia, signs of abdominal pain, and gastric reflux. The horse had undergone abdominal surgery at four months of age for a small intestinal volvulus involving most of the jejunum, and an end-to-end jejuno-ileal anastomosis was performed (Figure 1). The signs of abdominal pain developed the morning of presentation and had not responded to symptomatic treatment, including nonsteroidal anti-inflammatory medication (NSAID; flunixin meglumine, 1.1 mg/kg IV), fluid therapy (15 litres of Hartmann's solution IV), and gastric decompression through a nasogastric tube.

On a physical examination, the horse was quiet with a heart rate of 48 beats/min, respiratory rate of 15 breaths/min, and body temperature of 38.5 °C. The abdominal borborygmi was decreased at both sides and capillary refill time was delayed (>3 sec) along with congested mucous

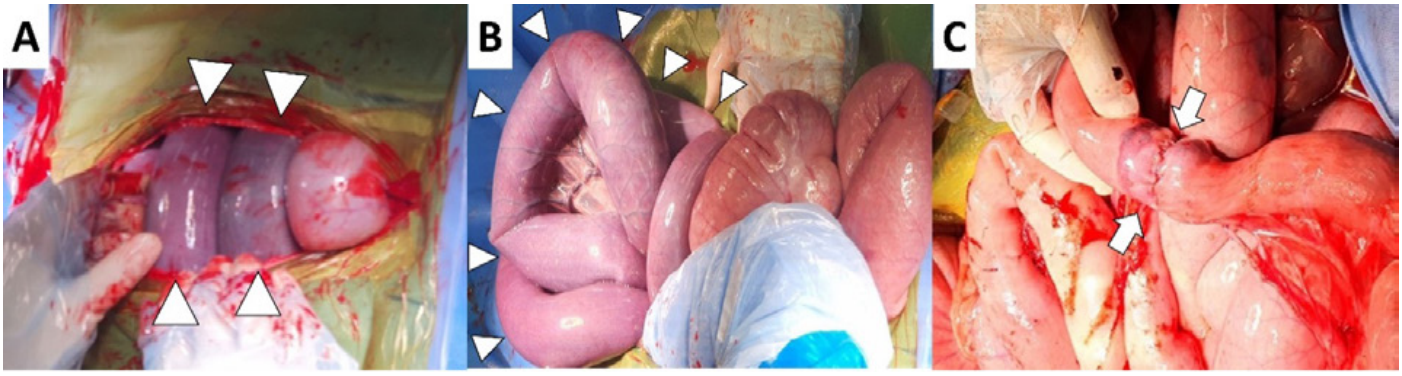

Figure 1: Detailed small intestine found on the first surgery at four-month of age. The strangulated small intestine was identified (A, B; arrowheads) and end-to-end jejuno-ileal anastomosis was performed (C; arrows)

membranes. A rectal examination was unremarkable. The hematology was within normal ranges. Serum biochemical analysis revealed elevated lactate (3.6 mmol/l, reference range, 1–1.5 mmol/l), hyperglycemia (156 mg/dl; reference range, 65–110 mg/dl), elevated creatinine kinase (2,003 U/l; reference range 120–470 U/l), decreased calcium (10.8 mg/dl, reference range; 11.5–14.2 mg/dl) and globulin (2.7 g/dl; reference range, 2.7–5.0 g/dl) levels. Abdominal ultrasonography revealed duodenal dilation (oval-shaped, 3.78 cm × 7.55 cm) with intraluminal fluid accumulation in almost the entire duodenal segment on the right side (Figure 2A).

An exploratory laparotomy was performed under general anesthesia. Antibiotic treatment included procaine penicillin G plus dihydrostreptomycin suspension (0.06 ml/kg IM, PPS injection, Daesung microbiological labs, Euiwang, Korea), gentamicin sulfate (6.6 mg/kg IV, Eagle gentamycin injection, Eaglevet, Seoul, Korea). NSAID was not given due to the administration prior to referral. The horse was premedicated with detomidine (0.02 mg/kg IV, Provet Detomidine®, Provet, Istanbul, Turkey). General anesthesia was induced with diazepam (0.1 mg/kg IV, Samjin pharm, Seoul, Korea) and ketamine (2.2 mg/kg IV, Yuhan Ketamine, Yuhan, Seoul, Korea) and maintained with isoflurane (Ifran, Hana pharm, Hwaseong, Korea) in oxygen through a closed circle ventilation system after endotracheal intubation. After clipping and aseptic preparation, a ventral midline incision was made. The abdominal exploration revealed the small intestine to be fluid-distended, which was related to the ileal obstruction (Figure 2B). The lumen of a segment of distal ileum was narrowed by a circumferential adhesion of the greater omentum, which caused a mechanical obstruction (Figure 3A). The cecum and large colon distal to the ileum collapsed with few palpable contents (Figure 2B). The ileum was released through a transection of the adhesion and omentectomy using a blunt dissection and an ultrasonic surgical instrument (Medisonic DU-137, Daiwha, Wonju, Korea) (Figure 3B, 3C). After the adhesionolysis, the remaining small intestine was inspected grossly. The color of the small intestine improved, and good motility was

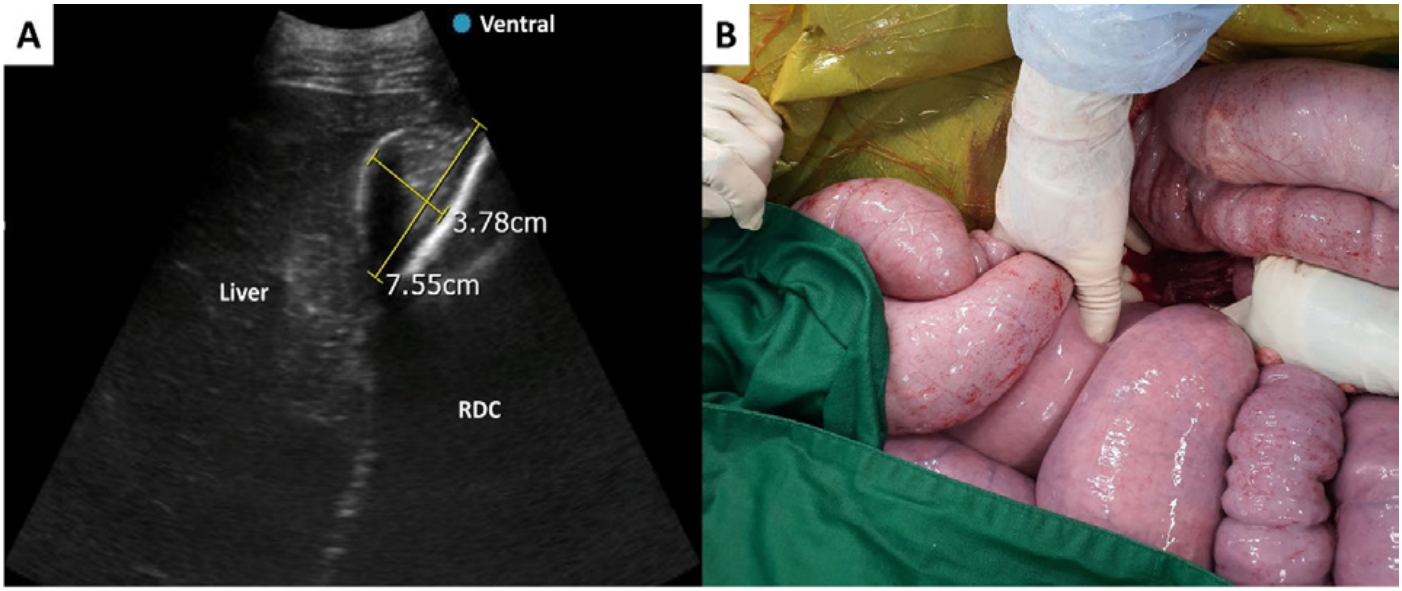

Figure 2: Ultrasonographic image (A) and gross appearance (B) of the distended small intestine in this study. (A) The duodenum was markedly dilated (oval-shaped, 3.78 cm × 7.55 cm) with an intraluminal fluid accumulation. The image was obtained from the right abdomen; RDC, and right dorsal colon. (B) The whole of the small intestine was grossly fluid distended which was related to the ileal obstruction

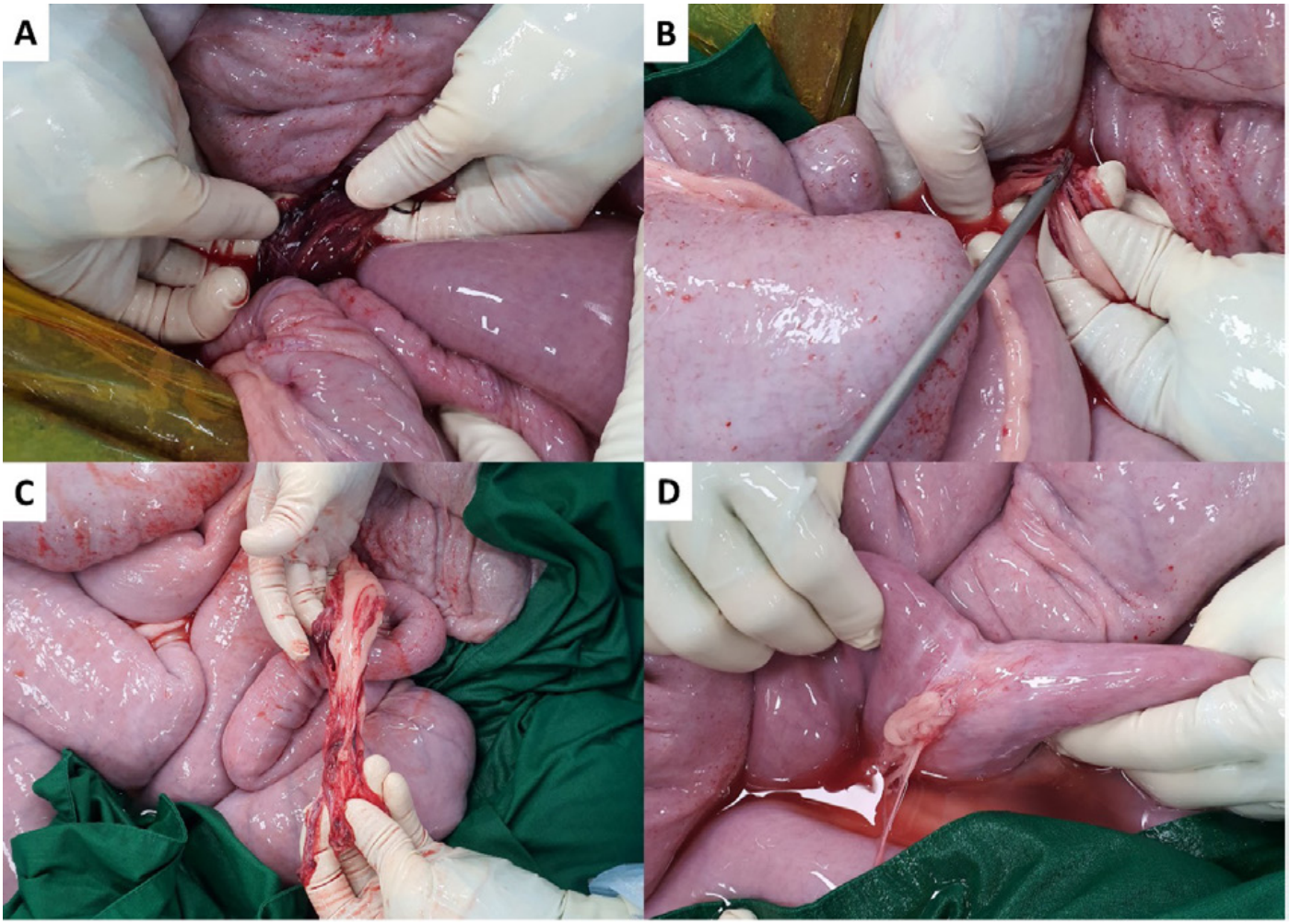

Figure 3: Identification of omental adhesion (A), adhesionolysis (B, C), and remnant secondary adhesion (D) in this study. (A) The distal ileum is encircled by the adhesion of the greater omentum. Both the mechanical obstruction and partial strangulation were caused by adhesion. (B) Omentectomy was performed using blunt dissection and an ultrasonic surgical instrument. (C) Resected omentum and relieved small intestines. The color and movement of the intestines gradually returned to normal. (D) Remnant adhesion at the anastomotic site of the previous surgery

observed throughout the intestine. The fluid distension was relieved by stripping the small intestinal contents into the cecum. Serosal scarring and adhesions were noted at the anastomotic site of the previous surgery (Figure 3D). Prior to closure, the abdominal cavity was lavaged with 15 L of heparinized saline (5,000IU/L). The linea alba, subcutaneous tissues, and skin were closed routinely.

Postoperatively, the horse recovered uneventfully from anesthesia. Postoperative medications included a continuous infusion of lidocaine (1.3 mg/kg IV as a bolus followed by a 0.05 mg/kg/min infusion) for the first 12 hours, flunixin meglumine (1.1 mg/kg IV once daily, Finadyne, MSD, France), procaine penicillin G (22,000 IU/kg IM once daily, PPS injection, Daesung microbiological labs, Euiwang, Korea), gentamicin sulfate (6.6 mg/kg IV once daily, Eagle gentamycin injection, Eaglevet, Seoul, Korea), famotidine (0.3 mg/kg IV twice daily, Gaster, Dong-A ST, Seoul, Korea), and omeprazole (4 mg/kg PO once daily, Abgard, Abler Pharmaceuticals, Houston, TX, USA) were administered for five days. Maintenance intravenous fluid of lactated Ringer's solution (60ml/kg/day) was infused for five days. After confirming the absence of complications such as postoperative ileus, gastric reflux, dehydration, and diarrhea, the infusion was discontinued. The horse showed hypocalcemia and hyperkalemia pre- and postoperatively (Table 1). Calcium gluconate (0.1 ml/kg of the 45% solution) and 50% dextrose were added to the fluid to improve the electrolyte imbalances. Food was re-introduced 24 hours postoperatively. Initially, the horse was provided with 0.5 kg of fresh grass four times daily, with a steady increase in the volume of feedings. Grass hay (0.5 kg, four times daily) was added to the fresh grass 48 hours after surgery and increased gradually to the full ration over the next five day. An abdominal bandage was maintained for two weeks. Probiotics (Lactobacillus spp., Enterococcus spp., and Saccharomyces boulardii) were added to the feeding during hospitalization. Short periods of hand-walking and grazing were allowed two or three times daily.

Serial ultrasonography revealed no small intestinal dilation and normal intestinal motility. Serial hematology showed elevated serum amyloid A (SAA; 2,063 mg/l; reference range < 20 mg/l), creatinine kinase (CK, 4,892 U/l; reference range

Table 1: Postoperative blood examination results obtained while hospitalized. The factors important for a prognosis evaluation and out of the normal range were specified. Blood biochemical analyses were performed using the VS2 (Abaxis, USA) for CK, AST, TBIL, BUN, Creatinine, Calcium, and Potassium; the Accutrend Plus (Roche, Germany) for Lactate; and the Solo (EuroLyser, Austria) for SAA

	Reference range	Preoperation	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7	Day 11
SAA (mg/l)	< 20	NA	2,063	1,912	1,502	1,112	NA	116.7	< 10
CK (U/l)	120–470	2,003	4,892	4,106	2,186	1,283	956	524	317
AST (U/l)	175–340	399	1,458	1,750	1,531	1,486	1,258	960	655
TBIL (mg/dl)	0.5–2.3	1.8	3.4	3.0	1.7	1.7	1.4	1.0	0.8
Lactate (mmol/l)	1–1.5	3.6	2.8	2.1	2.1	1.5	1.1	NA	NA
BUN (mg/dl)	7–25	23	15	11	12	13	14	12	14
Creatinine (mg/dl)	0.6–2.2	1.5	1.1	1.1	1.0	1.2	1.2	0.7	1.0
Calcium (mg/dl)	11.5–14.2	10.8	10.6	12.3	12.5	12.7	12.4	12.0	11.8
Potassium (mEq/L)	2.5–5.2	3.8	4.5	5.8	6.0	5.7	4.7	4.3	3.3

SAA, serum amyloid A; CK, creatinine kinase; AST, aspartate aminotransferase; TBIL, total bilirubin; BUN, blood urea nitrogen NA, not available

120–470 U/l), aspartate aminotransferase (AST, 1,750 U/l; reference range 175–340 U/l), and mild electrolyte imbalances (hypocalcemia and hyperkalemia) but the levels returned to the normal ranges with postoperative treatment within two weeks (Table 1). There was mild edema around the abdominal incision, but this resolved. During hospitalization, the horse showed no signs of abdominal pain and a good appetite. The staples were removed from the abdominal incision on day 15 after surgery and the horse was discharged. Follow-up at 1 year after surgery the horse remained healthy without any clinical signs of gastrointestinal disease.

Discussion

Postoperative intraabdominal adhesions are not an uncommon complication consequence of abdominal surgery (4, 5). Normally, the fibrinolytic capacity in the peritoneum exceeds the coagulation response so, abdominal adhesions do not occur under normal conditions (4). An imbalanced fibrinolytic system induced by specific causes, such as surgery and inflammation, damage to the mesothelial layer of the intestine, and reduced fibrinolytic activity, increases the risk of PIA (6). The earlier development of clinical adhesions is related to a poorer prognosis for survival (5). In equine medicine, adhesions are more often associated with surgical trauma rather than the site of the primary lesion, resection, or endotoxemia (7). Pathological adhesions causing postoperative abdominal pain and intestinal obstruction have been reported in 9% to 27% of horses (2), and small intestinal strangulating lesions associated with a greater omentum were present in 2.3% (32/1413) of horses undergoing exploratory laparotomy for abdominal pain (3). The potential causes of SIO in horses include pedunculated lipoma, herniation, epiploic foramen entrapment, incarceration within a mesenteric or omental rent, gastrosplenic ligament entrapment, volvulus, and adhesions (5). In the present case, based on the physical examination and blood analysis, differential diagnoses were listed as small intestinal enteritis, impaction, obstruction, and strangulating intestinal lesions. The horse showed a sudden onset of gastric reflux and hyperlactatemia, associated with small intestinal ileus and poor tissue perfusion, respectively. Furthermore, ultrasonographic duodenal dilation, delayed CRT, color change of the mucous membranes, and hypocalcemia supported the differential diagnosis. Given the clinical findings, small intestinal strangulation and obstruction were considered as the likely cause, and OA-associated SIO was confirmed during surgery.

Statistically, significant associations between the PIA rate and the following factors were identified in previous studies: surgical techniques, perioperative medications, and protective tissue coating solutions (1). Among the surgical techniques available, omentectomy has been advocated to reduce PIA (1, 3). In the present case, an omentectomy was not performed at the first abdominal surgery. As shown in this study, the omentum may lead to OA-related SIO, and omentectomy may be effective in preventing OA lesions. Broad-spectrum antimicrobials, NSAID, prokinetic agent, and intraperitoneal heparin were administered peri- and postoperatively in this study to minimize peritoneal and serosal inflammation. In addition, protective tissue coating solutions, such as hyaluronic acid (HA) and carboxymethylcellulose (CMC), resorbable membranes, and postoperative

abdominal lavage could have been used to prevent postoperative adhesions (1).

Lidocaine was administered postoperatively (1.3 mg/kg IV as a bolus followed by a 0.05 mg/kg/min infusion) and was discontinued after confirming that the horse showed no signs of postoperative ileus, including gastric reflux on nasogastric intubation and abnormalities on abdominal ultrasonography. The horse showed no abdominal pain with normal intestinal borborygmi and good appetite toward to the hang hay nets outside the stall. Lidocaine is often used clinically as a constant rate infusion (CRI) for its potential analgesic, anti-inflammatory, and prokinetics properties (2). Although the prokinetic efficacy of lidocaine is still controversial (2, 8), there are evidence that lidocaine improves postoperative reflux, hospitalization duration, time to the first fecal passage, and survival rate (9-12). In a meta-analysis of the recent literature, the use of lidocaine in horses undergoing gastrointestinal surgery for the small intestinal disease was associated with an increased survival rate (8). In addition, surveys showed that most (79%) ECVS/ECEIM and 68% of ACVS/ACVIM, and ACVECC diplomates use lidocaine to treat postoperative ileus (13, 14).

On admission, the horse showed elevated serum CK and AST concentrations pre- and postoperatively (Table 1). Elevated CK and AST activities in horses with colic are frequently associated with muscle trauma caused by lying down, thrashing, rolling, and trailering to hospitals (15). Evidence of hepatic and metabolic changes associated with CK and AST in surgical colic patients has also been reported (16). The horse in this study showed a significantly higher CK value postoperatively (Table 1). This was possibly attributed to the decreased renal and intestinal blood flows, which were less than 50% of the awake value during deep anesthesia, causing significant CK and AST elevations on the day after anesthesia (17, 18).

In addition to the anti-inflammatory and prokinetic drug use, supportive care was conducted to enhance the postoperative prognosis. Maintenance intravenous fluid of lactated Ringer's solution was infused for five days. The horse showed hypocalcemia and hyperkalemia pre- and postoperatively (Table 1). Calcium gluconate and 50% dextrose were added to the fluid to improve electrolyte imbalances. The horse received probiotics (Lactobacillus spp., Enterococcus spp., and Saccharomyces boulardii) for normal microbial flora restoring and protective benefits of intestinal pathogenic bacteria. Gastric protectants, such as omeprazole and famotidine, were administered after surgery to prevent gastric ulcers. Hospitalized colic patients are frequently at increased risk of developing gastric ulcers associated with the administration of NSAID and treatment, stress, and feed deprivation (19). The highly effective treatment of gastric ulcers using omeprazole is well described (2, 19). The anti-ulcer medication was discontinued after restoring normal feeding and appetite with no gastric reflux. Although gastric protectants were used to prevent postoperative gastric ulcers in this study, the potential risks associated with omeprazole administration, such as rebound gastric hyperacidity at the time of discontinuation, decreased calcium absorption during administration, and gastrointestinal toxicity related to the concurrent administration with NSAIDs, have been suggested (20).

Conclusions

This case report describes a secondary SIO associated with an OA after end-to-end jejuno-ileal anastomosis in a thoroughbred horse. The obstruction was surgically resolved by transecting the adhesion and performing an omentectomy. These findings highlight the importance of recognizing and addressing OA as a potential cause of SIO after intestinal surgery in horses, underscoring the importance of preventing postoperative adhesions and application of appropriate surgical techniques.

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Primer sekundarna obstrukcije tankega črevesa, povezane z adhezijo omentuma po jejuno-ilealni anastomozi pri čistokrvnem konju

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Izvleček: 19 mesečna čistokrvna kobila je imela akutno abdominalno bolečino. Pri starosti 4 mesecev so ji med abdominalno operacijo odstranili večino zadrgnjenega jejunuma in ileuma, ki so ju anastomozirali s tehniko konec s koncem. Pri kobili so nato diagnosticirali obstrukcijo ileuma kot posledico adhezije velikega omentuma, ki je vodila v mehansko zaporo lumna distalnega ileuma. Ileum je bil sproščen s prerezom adhezije in izvedbo omentektomije. Po kirurškem posegu je kobila hitro okrevala in bila po petnajstih dneh odpuščena iz veterinarske oskrbe. Ta klinični primer opisuje obstrukcijo ileuma zaradi adhezije omentuma po jejuno-ilealni anastomozi pri čistokrvni kobili. Opisani so klinični, slikovni in kirurški izvidi.

Ključne besede: kolika; konj; jejuno-ilealna anastomoza; adhezija omentuma; obstrukcija tankega črevesa

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