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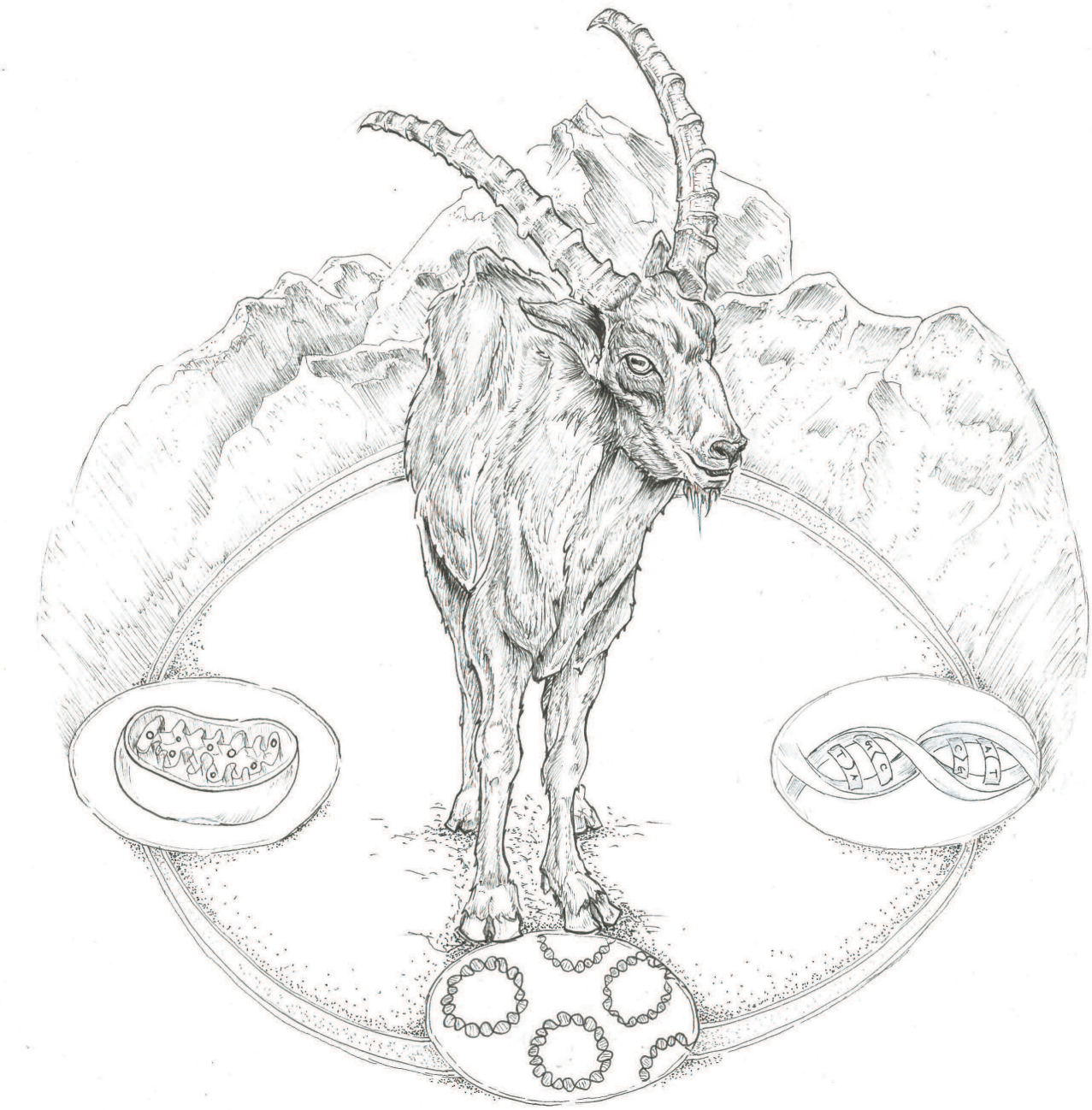
Slovenski veterinarski zbornik

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA



ISSN 1580-4003

Volume 60, Number 3, Pages 115–172

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Volume 60, Number 3, Pages 115–172

The Scientific Journal of the Veterinary Faculty University of Ljubljana

Slovenian Veterinary Research
Slovenski veterinarski zbornik

Previously: RESEARCH REPORTS OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA
Prej: ZBORNIK VETERINARSKE FAKULTETE UNIVERZA V LJUBLJANI

4 issues per year / Izhaja štirikrat letno
Volume 60, Number 3 / Letnik 60, Številka 3

Published by / Založila	University of Ljubljana Press / Založba Univerze v Ljubljani
For the Publisher / Za založbo	Gregor Majdič, Rector of the University of Ljubljana / Rektor Univerze v Ljubljani
Issued by / Izdala	Veterinary Faculty University of Ljubljana / Veterinarska fakulteta Univerze v Ljubljani
For the Issuer / Za izdajatelja	Breda Jakovac Strajn, Dean of the Veterinary Faculty / Dekanja Veterinarske fakultete
Address	Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov	Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
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Sponsored by / Sofinancira	The Slovenian Research Agency / Javna agencija za raziskovalno dejavnost Republike Slovenije
Printed by / Tisk	DZS, d.d., Ljubljana, September 2023
Number of copies printed / Naklada	220
Indexed in / Indeksirano v	Agris, Biomedicina Slovenica, CAB Abstracts, IVSI Urlich's International Periodicals Directory, Science Citation Index Expanded, Journal Citation Reports – Science Edition
	https://www.slovetres.si/
	ISSN 1580-4003

Cover illustration by Pšenica Kovačič depicts the Alpine ibex against the backdrop of iconic Slovenia's Triglav mountain and is inspired by the manuscript by Bužan et al. in this issue. See page 161.

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DOI 10.26873/SVR-1872-2023
UDC 639.111.2:601.4:575:364.692:574.1(497.4)
Pages: 119–21

Editorial

Reviving the Alpine Ibex: Addressing Genetic and Health Concerns of Slovenian Ibex with Broader Implications in Biodiversity

Reševanje alpskega kozoroga: Genetski in zdravstveni problemi slovenskih kozorogov s širšimi posledicami za biodiverziteto

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Accepted: 15 September 2023

This issue's cover illustration depicting the Alpine ibex against the backdrop of iconic Slovenia's Triglav mountain is inspired by the manuscript by Bužan et al. published in the current issue and serves as a poignant reminder of a lost legacy and biodiversity due to greed and overhunting. These magnificent creatures thrived entire European Alpine region before their extermination in the 19th century, with the only surviving wild population of Alpine ibex in the Gran Paradiso National Park (then a royal hunting reserve) in Italy. Reintroduction initiatives from this small population have since rebounded ibex to tens of thousands across the Alpine region. However, as the article on the Slovenian population of Alpine ibex by Bužan et al. in this issue clearly shows, while current numbers seem more promising, the genetic bottleneck they experienced from this near-extinction event has left lasting impacts on their genetic diversity.

Biodiversity is decreasing worldwide due to factors such as habitat destruction, pollution, climate change, and overexploitation of species. Additionally, low genetic diversity within populations makes species more vulnerable to disease, environmental changes, and other threats, further exacerbating the decline. Lost genetic diversity in the Alpine ibex

Ilustracija na naslovnici te številke, ki prikazuje Alpskega kozoroga pred ikonično goro Slovenije, Triglavom, je navdihnjena po članku Bužan s sod., objavljenem v tej številki, in služi kot opomin izgubljeni dediščini in biodiverziteti zaradi pohlepa in prekomernega lova. Te veličastne živali so uspevale v celotni evropski Alpski regiji, preden so bile iztrebljene v 19. stoletju, z edino preživelo divjo populacijo v Narodnem parku Gran Paradiso (takrat kraljevi lovski rezervat) v Italiji. Ponovne naselitve iz te majhne populacije so od takrat od danes dvignile število kozorogov na deset tisoče v celotni Alpski regiji. Vendar, kot članek o slovenski populaciji Alpskih kozorogov avtorice Bužan s sod. jasno kaže, čeprav se trenutna številka zdi obetavna, pa je genetsko ozko grlo, nastalo zaradi prekomernega lova, pustilo trajne posledice na njihovi genetski raznolikosti.

Biodiverziteta se po vsem svetu zmanjšuje zaradi dejavnikov, kot so uničevanje habitata, onesnaževanje, podnebne spremembe in prekomerno izkoriščanje vrst. Poleg tega nizka genetska raznolikost znotraj populacij povzroči, da so vrste bolj ranljive za bolezni, okoljske spremembe in druge faktorje. Izguba genetske raznolikosti pri Alpskem kozorogu ni več tematika za akademske razprave ali politično

is far from being a mere academic consideration or a politically attractive topic. It has tangible and dire repercussions. This isn't just about yet another genetic screening project exemplified in the article of Bužan et al. It affects the health and survival of this species and has negative consequences on the diversity and health of the ecosystem as a whole. A limited genetic pool cripples the ibex's ability to ward off even the mildest of microbial and parasitic infections. The Scabies mite, causing Sarcoptic mange, serves as a glaring example. In a robust and genetically diverse population, such a parasite might cause concern but not alarm.

Yet, for the genetically inbred Alpine ibex, it represents a profound threat, intensifying concerns for both their conservation and management. This is not about genetic nuances; it's a life-and-death struggle influenced directly by their genetic resilience, or lack thereof.

Veterinarians have been at the forefront of combating Sarcoptic mange in wild Alpine ibex populations. As the condition progresses, afflicted animals experience immense suffering, enduring severe dehydration, weight loss, and secondary infections, ultimately leading to a painful and protracted demise. Veterinarians' efforts span from field surveillance, and diagnostics to direct interventions, such as treating afflicted individuals and preventive measures for those at risk. Modern veterinary medicine does offer a range of treatments, from antiparasitic medications to immune boosters. However, the challenge lies in administering these treatments to wild populations, ensuring they reach those most in need, and assessing their long-term efficacy. However, the rugged, steep, and rocky Alpine terrains make it almost impossible for veterinarians to reach and treat afflicted ibex directly, exacerbating their prolonged suffering before their agonizing end. The real efficacy lies not just in treating the symptoms but in fortifying the ibex's natural genetic defenses. This emphasizes, once again, the profound importance of genetic diversity, as a diverse gene pool would naturally equip the ibex with a better defense mechanism against such diseases.

While molecular methods have elucidated the genetic plight of many populations, Slovenian ibex remained unstudied until now. Our analysis of the mitochondrial DNA for both neutral and adaptive loci of ibex from Slovenian Julian and Kamnik-Savinja Alps revealed a startling lack of genetic diversity. The two Slovenian populations possess just one mtDNA haplotype and a single functional allele for MHC DRB exon 2, emphasizing the dire need for diversity reintroductions. Regulatory constraints label the ibex as non-native in Slovenia, hindering conservation. Regrettably, the narrative of overhunting echoes loudly here. These majestic creatures were hunted to oblivion in several regions, only to face the current plight of being treated as 'non-native' in lands they once roamed freely. Given current findings of genetic depletion, there's an urgent call for a policy shift to recognize the ibex as native to Slovenia, facilitating vital conservation actions. International and interdisciplinary

privlačna tema. Ima oprijemljive in hude posledice. Tu ne gre le za genetske karte ali še en genetski presejalni projekt, kot je prikazan v članku Bužan s sod.. Izguba genetske biodiverzitete vpliva na zdravje in preživetje te vrste in ima negativne posledice za raznolikost in zdravje celotnega ekosistema. Nizka genetska raznolikost onemogoča kozorogu, da se obrani pred celo najšibkejšimi mikrobnimi in parazitskimi okužbami. Primer takšne okužbe je s pršico Sarcoptes scabiei, ki povzroča garje. V robustni in genetsko raznoliki populaciji bi takšen parazit morda povzročil zaskrbljenost, vendar ne bi povzročil alarma. Vendar pa za genetsko osiromašene kozoroge to predstavlja resno grožnjo za njihovo ohranitev in upravljanje s populacijo. Tu gre dejansko za boj za življenje in smrt, kjer je vpletena njihova genetska odpornost, ali bolje rečeno, pomanjkanje le-te.

Veterinarji so na čelu boja proti povzročiteljici garij pri divjih populacijah kozorogov. Ko se stanje poslabša, prizadete živali doživljajo veliko trpljenja, prenašajo hudo dehidracijo, izgubo telesne mase in sekundarne okužbe, kar na koncu vodi v bolečo in dolgotrajno smrt. Prizadevanja veterinarjev segajo od terenskega nadzora in diagnostike do neposrednih intervencij, kot je zdravljenje prizadetih živali in preventivni ukrepi za tiste, ki so ogroženi. Sodobna veterinarska medicina ponuja različne metode zdravljenja, od zdravil proti parazitom do imunskih stimulantov. Toda izziv leži v upravljanju teh zdravljenj na divjih populacijah, zagotavljanju, da dosežejo tiste, ki jih najbolj potrebujejo, in monitoring njihove dolgoročne učinkovitosti. Skalnat in strm alpski teren skorajda onemogoča veterinarjem, da bi neposredno dosegli in zdravili prizadete kozoroge, kar še poslabša njihovo dolgotrajno trpljenje, preden doživijo svoj mučen konec. Prava učinkovitost ni le v zdravljenju simptomov, temveč tudi v utrjevanju naravnega genetskega obrambnega mehanizma kozoroga. To še enkrat poudarja pomen genetske raznolikosti, saj bi bolj raznolik genski bazen naravno opremil kozoroge z boljšim imunskim odzivom proti takšnim boleznim.

Čeprav so molekularne metode razjasnile genetsko strukturo mnogih populacij, slovenski kozorogi doslej še niso bili raziskani. Naša analiza mitohondrijske DNA za nevtralne in adaptivne genetske lokuse kozorogov iz slovenskih Julijskih in Kamniško-Savinjskih Alp je razkrila presenetljivo pomanjkanje genetske raznolikosti. Obe slovenski populaciji imata le en mtDNA haplotip in en funkcionalen alel za MHC DRB ekson 2, kar poudarja nujno potrebo po oplemenjevanju. Regulativne omejitve označujejo kozoroge kot tujerodne v Sloveniji, kar omejuje ohranjanje biodiverzitete. Da se jih danes obravnava kot 'tujerodne' je nesmiselno že v luči poznavanja zgodovine o prekomernem lovu v slovenskih Alpah. Glede na trenutne ugotovitve nizke genetske raznolikosti je nujen poziv k spremembi politike in priznanju kozorogov kot domorodnih v Sloveniji, kar bo omogočilo ključne nadaljnje ukrepe za ohranjanje. Usoda Alpskega kozoroga služi kot še en opomin na zapleteno prepletenost med zgodovino, genetiko, zdravjem živali in politiko ohranjanja. V tem

cooperation, thorough health screenings, and community involvement will be essential to this endeavor. The destiny of the Alpine ibex serves as yet another reminder of the intricate bond between history, genetics, animal health, and conservation. At this crucial juncture, we appeal to policymakers, conservationists, and the public at large.

In the end, it may be appropriate to mention a Slovenian Folk tale, "Zlatorog" (Goldhorn), featuring an Alpine ibex with golden horns as a protector of natural land and an indicator of how greed and ambition can ruin the environment. Originally written by Dežman and published in the "Laibacher Zeitung" in 1868, this story has been integral in Slovenian culture and has been retold and popularized by many, most notably by the poet Rudolf Baumbach in his ballad "Zlatorog," translated in many languages. The story revolves around a hunter who seeks the treasure hidden in the Triglav mountains, guarded by Goldhorn. Driven by jealousy, greed, and ambition, the hunter shoots Goldhorn to be able to get to the treasure. However, as the Goldhorn bleeds, he eats the magical Triglav rose, his horns start glowing with intense golden light to dazzle the hunter and drive him over the cliff to his tragic end. In his fury, Goldhorn transformed a once fertile and biodiverse mountainous region into a barren expanse of stone scree. This can be seen as a cautionary tale: if we mistreat and exploit nature, it may reach a tipping point of imbalance and self-destruction. The ancient tale of Goldhorn is a profound allegory that underscores the perils of unbridled greed and ambition juxtaposed against the sanctity of nature.

This intrinsic value of the natural world is one that beckons a resurgence through dedicated research, such as the insights offered in the mentioned article. Interdisciplinary research is crucial in preserving biodiversity and maintaining healthy ecosystems, as it integrates diverse perspectives and methodologies to comprehensively address complex environmental challenges. Only through a collaborative approach, drawing from multiple disciplines, can we truly understand and effectively protect the intricate web of life on our planet. Yet, it's not just research that holds the key. Our moral compass and policy directions play an equally pivotal role. Conserving the ibex and their pristine habitats isn't a mere environmental prerogative; it's a testament to our shared commitment to the future. In protecting them, we don't just ensure our own survival but also uphold a shared legacy of the legend that binds man, wildlife to ensure the balance in the nature.

ključnem trenutku pozivamo odločevalce, strokovne organizacije in širšo javnost h ukrepanju.

Na koncu bi bilo morda primerno omeniti slovensko ljudsko legendo »Zlatorog,« v kateri nastopa Alpski kozorog z zlatimi rogovi kot zaščitnik naravnega okolja in kazalnik, kako človekova nezmernost uničujeta okolje. Zgodbo je prvotno napisal Dežman in objavil v »Laibacher Zeitung« leta 1868. Ta zgodba je postala del slovenske kulture in jo je veliko ljudi povzelo in populariziralo. Najbolj znana je pesnitev Rudolfa Baumbacha »Zlatorog,« ki je prevedena v mnoge jezike. Zgodba se vrti okoli lovca, ki išče zaklad, skrit v Triglavskih gorah, ki ga varuje Zlatorog. Poganjan z ljubosumjem in pohlepom o, je lovec ustrelil Zlatoroga, da bi prišel do zaklada. Toda ko Zlatorog krvavi, poje čarobno Triglavsko rožo, njegovi rogovi začnejo svetiti z intenzivnimi zlatimi žarki, ki oslepi lovca, da le ta omahne čez rob skalne police do tragičnega konca. V svoji jezi je Zlatorog nekoč rodovitno in biodiverzitetno gorsko regijo spremenil v pusto kamnito pokrajino. To se lahko razume kot opozorilna zgodba: če naravo zlorabljamo in izkoriščamo, lahko doseže točko neravnovesja in samouničenja. Stara zgodba o Zlatorogu je globoka alegorija, ki poudarja nevarnosti neukrotljive človekove sebičnosti, nasproti ravnovesju narave.

Interdisciplinarno raziskovanje je ključno pri ohranjanju biotske raznovrstnosti in vzdrževanju zdravih ekosistemov, saj integrira različne perspektive in metodologije za celovito obravnavanje kompleksnih okoljskih izzivov. Le preko sodelovanja, ki črpa znanje iz več disciplin, lahko resnično razumemo in učinkovito zaščitimo zapleteno mrežo življenja na našem planetu. Vendar pa ni le raziskovanje tisto, ki zagotavlja preživetje. Naš moralni kompas in usmeritve politike igrajo enako pomembno vlogo. Ohranjanje kozorogov in njihovih neokrnjenih habitatov ni le okoljska dolžnost; to je dokaz naše skupne zavezanosti prihodnosti. S tem, ko jih ščitimo, ne zagotavljamo le svojega preživetja, ampak tudi uresničujemo skupno dediščino, ki, kot pravi legenda, zahteva sobivanje človeka in živali za zagotavljanje ravnotežja v naravi.

DOI 10.26873/SVR-1879-2023
UDC 577.2:615.371:06.05NOBEL:172.4
Pages: 123–26

Editorial

In the Spotlight

A 2023 Nobel Prize in Physiology or Medicine: Pathway for Next Generation of Vaccines

Nobelova nagrada 2023 za fiziologijo ali medicino: pot do naslednjega rodu cepiv

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Accepted: 28 September 2023

This year's Nobel Prize in Physiology or Medicine has been awarded to Katalin Karikó and Drew Weissman for discoveries that enabled the development of messenger RNA (mRNA) vaccines against COVID-19. mRNA is a transient molecule in the cell that conveys the instructions for synthesis of a protein from the nucleus, where instructions are stored as a genetic code in the DNA, to the cell's protein making machinery (ribosomes) in the cytoplasm. It took several decades of research to uncover how mRNA could be used to deliver an antigen into cells and trigger the body's own immune response.

Traditional vaccine development, which used a weakened or dead virus to stimulate an immune response against the disease, is lengthy and costly. Progress in molecular biology enabled the development of vaccines based on individual viral components, where parts of the viral genetic code are used to make proteins that stimulate the formation of virus-blocking antibodies. The most recently developed mRNA vaccines contain viral mRNA that, when injected into the body, instructs the cells to produce parts of viral proteins that trigger the immune response. Since mRNA can be quickly synthesized and modified, the development of mRNA vaccines can be much faster than traditional vaccines. By discovering how to make mRNA stable and prevent immune activation by the mRNA itself, the seminal discoveries of his year's Nobel Prize laureates were essential to the development and implementation of mRNA vaccines.

Letošnja Nobelova nagrada za fiziologijo ali medicino je bila podeljena Katalin Karikó in Drewu Weissmanu za odkritja, ki so omogočila razvoj cepiv proti COVID-19 na osnovi sporočilne RNA (mRNA). mRNA je prehodna molekula v celici, ki posreduje navodila za sintezo proteinov iz jedra, kjer so navodila shranjena kot genetski kod v DNA, celičnemu sistemu za izdelovanje proteinov (ribosomov) v citoplazmi. Potrebnih je bilo več desetletij raziskav, da bi odkrili, kako uporabiti mRNA za prenos antigenov celicam in začetek telesu lastnega imunskega odziva.

Tradicionalni razvoj cepiv, ki je uporabljal oslabljene ali mrtve viruse za stimulacijo imunskega odziva proti boleznim, je drag in dolgotrajen. Napredek v molekularni biologiji je omogočil razvoj cepiv na osnovi posameznih virusnih delov. Pri tem so deli virusnega genetskega koda uporabljeni za proizvodnjo proteinov, ki stimulirajo tvorbo protiteles proti virusom. Najsodobnejša cepiva na osnovi mRNA vsebujejo del virusne mRNA, ki ob injiciranju v telo celicam posredujejo navodila za proizvodnjo delov virusnih proteinov, ki sprožijo imunski odziv. Ker je lahko mRNA hitro sintetizirana in nadgrajena, je lahko tudi proizvodnja cepiv na osnovi mRNA precej hitrejša kot proizvodnja tradicionalnih cepiv. Z odkritjem, kako molekulo mRNA napraviti stabilnejšo in kako preprečiti imunsko aktivacijo s samo molekulo mRNA, so bila temeljna odkritja letošnjih Nobelovih nagrajencev, ključna za razvoj in uporabo cepiv na osnovi mRNA.

Gene therapy produces a therapeutic effect by genetically modifying cells through introduction of a new gene or reconstruction of existing defective genetic material. In the case of mRNA vaccines, the genetic code for an antigen (i.e., a viral protein) triggers an immune response that ultimately leads to immune protection against the virus. Gene therapy has been gaining momentum in human medicine as a versatile therapeutic approach in the past decade, following the first gene therapy approvals in Europe in 2012 and 2016 for Glybera (to reverse lipoprotein lipase deficiency) and Strimvelis [to treat severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID)], respectively (1, 2), and the first approval in the USA in 2017 for Kymriah (to treat acute lymphoblastic leukemia)(3). Besides offering hope for a cure to patients with rare diseases, certain types of cancer, and developmental genetic diseases, gene therapy also has enormous potential in preventing diseases via mRNA vaccines. In the post-genomic era, the development of technology and the availability of genomic data enables the rational and targeted design and clinical testing of various kinds of gene therapeutics.

Genes can be delivered to the cell of interest by DNA plasmids, viral vectors, mRNA, or other methods. Although gene transfer technology using mRNA was investigated for decades, several roadblocks, including a lack of mRNA stability and immune rejection of RNA molecules, hindered the efficiency of mRNA delivery. Through several decades of research, Katalin Karikó and Drew Weissman made vital discoveries that were instrumental in breaching these roadblocks. First, Karikó and Weissman discovered that the introduction of chemical modifications in mRNA nucleoside bases almost abolished inflammation when base-modified mRNA was delivered to dendritic cells (4). This discovery was a paradigm shift in our understanding of how cells recognize and respond to different forms of mRNA, as it showed that in vitro transcribed mRNA containing modified bases evades innate immune recognition. Additionally, they found that base modification of mRNA increased its stability and, consequently, protein production in cells (5, 6). These two discoveries, along with the unprecedented investment by the pharmaceutical industry, enabled the rapid development of the mRNA vaccine during recent pandemics and opened the flood doors for novel gene therapy opportunities, including vaccine development and cancer immunotherapy (7, 8).

Compared to the viral vectors currently used in most approved gene therapies, mRNA gene transfer is a safer alternative because mRNA is devoid of potentially toxic viral genes and regulatory elements present in the viral vectors. Furthermore, mRNA does not integrate into the genome, is non-replicative, and decays within a few days. This temporary therapeutic expression of the encoded protein is desirable in vaccine development and has been implemented in certain CAR-T therapy research applications (8, 9).

Genska terapija povzroči terapevtski učinek z genskim spreminjanjem celic z vnašanjem novih genov ali popravljanjem obstoječega, okvarjenega genetskega materiala. V primeru cepiv na osnovi mRNA genetski kod za antigen (npr. virusni protein) sproži imunski odziv, kar v končni fazi privede do imunske zaščite proti virusu. Genska terapija v zadnjem desetletju pridobiva na pomembnosti kot raznovrstni terapevtski pristop, po prvih odobritvah tovrstne terapije v Evropi leta 2012 in 2017 za Glybero (ki je zdravila pomanjkanje lipoproteinske lipaze) ter Strimvelis (ki zdravi hudo kombinirano imunsko pomanjkljivost zaradi pomanjkanja adenozinske deaminaze (ADA-SCID)) (1, 2) in po prvi odobritvi v ZDA za Kymriah (ki zdravi akutno limfoblastično levkemijo in ne-Hodgkinove limfome) (3). Poleg tega, da lahko ponudi upanje za ozdravitev bolnikom z redkimi boleznimi, nekaterimi oblikami raka in razvojnimi boleznimi, kaže genska terapija velikanski potencial pri preventivi bolezni s cepivi na osnovi mRNA. V po-genomski eri razvoj tehnologije in dostopnost genomskih podatkov omogočata racionalno in ciljno načrtovanje in klinično preizkušanje različnih oblik genske terapije.

Geni so lahko v ciljno celico dostavljeni s plazmidi DNA, z virusnimi vektorji, mRNA ter z drugimi metodami. Čeprav je bila tehnologija genskega prenosa z uporabo mRNA predmet raziskovanj že več desetletij, so številne ovire, vključno s pomanjkljivo stabilnostjo mRNA in imunskim zavračanjem molekul mRNA, zadrževale učinkovito dostavljanje mRNA v celico. Med večdesetletnimi raziskavami sta Katalin Karikó in Drew Weissman prišla do ključnih odkritij, nujnih za odpravo teh ovir. Najprej sta odkrila, da je uvajanje kemijskih sprememb v nukleozidne baze mRNA skoraj odpravilo vnetne procese, kadar je bila mRNA z modificiranimi bazami uvedena v dendritične celice (4). To odkritje je pomenilo premik v načinu razmišljanja, kako celice prepoznajo in odgovorijo na različne oblike mRNA. Ugotovila sta, da se in vitro prepisane mRNA, ki vsebujejo modificirane baze, izognejo prirojenemu imunskemu prepoznavanju. Poleg tega sta ugotovila tudi, da modifikacija baz pri mRNA poveča njeno stabilnost in posledično produkcijo proteinov v celici (5, 6). Ti dve odkritji sta, skupaj z do tedaj nepredstavljivimi investicijami v farmacevtski industriji, omogočili hiter razvoj cepiv na osnovi mRNA med nedavno pandemijo in na stežaj odprli vrata priložnostim novih genskih terapij, vključno z razvojem cepiv in imunoterapij raka (7, 8).

V primerjavi z virusnimi vektorji, ki so trenutno najpogosteje uporabljeni vektorji pri večini odobrenih genskih terapij, je genski prenos z mRNA varnejši pristop zato, ker mRNA ne vsebuje potencialno nevarnih virusnih genov in regulatornih elementov, ki se nahajajo v virusnih vektorjih. Poleg tega se mRNA ne vključuje v genom, se ne replicira in razpade v nekaj dneh. Tako začasno terapevtsko izražanje kodiranega proteina je zaželeno pri razvoju cepiv in je bilo uporabljeno tudi pri nekaterih oblikah razvoja terapij s CAR-T (8, 9).

V veterinarski medicini, tudi zaradi manj stroge zakonodaje v primerjavi s humano medicino, genska terapija ni nič

Due to the less stringent regulations in veterinary medicine compared to human medicine, gene therapy is not new in veterinary medicine. Rather, comparative medicine is leading the way in the development of novel approaches, such as combining gene therapy with electrochemotherapy in cancer treatment to improve the therapeutic outcome, a technique pioneered by and also mastered through the collaborative efforts of Slovenian veterinarians, medical doctors, and comparative researchers (10-13). In horses and dogs, interleukin (IL)-12 based gene therapy improved the electrochemotherapy antitumor effects on spontaneously occurring tumors in large and companion animals (10-13) and has potential in translating to human medicine. Furthermore, mRNA vaccines hold great promise in veterinary medicine. In past years, several mRNA vaccines have entered clinical trials. Due to their low risk of insertional mutagenesis, high potency, and potential for low-cost manufacturing, mRNA vaccines promise solutions to combat emerging and re-emerging infectious diseases, such as rabies, Zika, and influenza (14, 15)

The exciting discoveries leading to this year's Nobel Prize in Physiology or Medicine will impact future human and veterinary medicine and may be critical to help combat current and future zoonotic diseases.

Funding: K.F.T. is funded by the Texas Tech University start-up, Cancer Prevention and Research Institute (CPRIT) of Texas Scholar Award RR200059, the Foundation for Prader–Willi Syndrome (FPWR) Grants 22-0321 and 23-0447.

Acknowledgments: We thank Dr. Rebecca Gee for the language editing.

novega. Primerjalna medicina nas vodi v razvoju novih pristopov, kot je kombinacija genske terapije z elektrokemoterapijo raka za izboljšanje terapevtskega učinka, tehnik, ki so jih s skupnimi napori vzpostavili in vodili slovenski veterinarji, zdravniki in primerjalni raziskovalci (10–13). Pri konjih in psih je genska terapija z interlevkinom (IL)-12 izboljšala protitumorske učinke elektrokemoterapije pri spontanih tumorjih velikih živali in hišnih ljubljenčkov (10–13) in ima tudi potencial translacije v humano medicino. Poleg tega v veterinarski medicini veliko obljubljajo tudi cepiva na osnovi mRNA. Številna cepiva se v zadnjih letih že klinično preizkušajo. Zaradi nizkega tveganja za insercijsko mutagenezo, visokih zmogljivosti in potencialno nizkih stroškov proizvodnje cepiva na osnovi mRNA obetajo rešitve za boj proti novim in obstoječim kužnim boleznim, kot so steklina, zika in gripa (14, 15).

Vznemirljiva odkritja, ki so privedla do letošnje Nobelove nagrade za fiziologijo ali medicino, bodo vplivala na humano in veterinarsko medicino in bodo lahko tudi ključno pripomogla k boju z zoonozami danes in v prihodnosti.

Financiranje: K.F.T. je financirana s sredstvi 'Texas Tech University start-up', 'Cancer Prevention and Research Institute (CPRIT) of Texas Scholar Award RR200059' in s sredstvi 'Foundation for Prader–Willi Syndrome (FPWR) Grants 22-0321 and 23-0447'.

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DOI 10.26873/SVR-1291-2023
UDC 632.95.021.0:616.992:616-036.8:638.132(213.5)
Pages: 127–34

Original Research Article

In vitro effects of hydro-methanolic extract from *Gliricidia sepium* leaves on larvae of *Haemonchus contortus*

Key words

flavonoids;
flavonols;
larvae;
nematodes;
tannins

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Abstract: The aim of this in vitro study was to evaluate the anthelmintic effects of extracts of *Gliricidia sepium* on sheathed and exsheathed larvae of *Haemonchus contortus*. Larvae of this parasite were incubated at 20–25 °C in hydro-methanolic extracts of leaves from this tropical tree at concentrations of 12.5, 25, 50, 100, and 200 mg/mL for 24, 48, or 72 h. Water and ivermectin were negative and positive controls, respectively. Total phenolic compounds of leaves of *G. sepium* were 6.4 ± 2.4 mg/g of dry matter. Other compounds identified in this leguminous tree by HPLC-mass spectrometry and that may be responsible for the anthelmintic effects observed were vanillin 4-sulfate, prodelphinidin p-coumaroyl glucose, kaempferol 3-o-glucosyl-rhamnosyl-glucoside, kaempferol-3-O-xylosyl rutinoside, p-coumaric acid, luteolin 7-rutinoside, isorhamnetin 3-glucoside-7-rhamnoside, and dihydro ferulic acid. At doses of 100 mg/mL mortality rate of sheathed and exsheathed *H. contortus* was 21.6 and 44.7%, respectively for 72 h of incubation. At 200 mg/mL, the hydro-methanolic extracts of *G. sepium* killed 61.5 and 93.8% of sheathed and exsheathed larvae, respectively, after 72 h of incubation. The effective concentration of the *G. sepium* extract for 50% sheathed and exsheathed larvae mortality (EC50) after 72 h of incubation was 74 mg/mL (CI = 46–100) and 68 mg/mL (CI= 32–100), respectively. The significant (P<0.001) ability to kill larvae compared to the negative controls, suggests in vitro anthelmintic properties of *G. sepium* against *H. contortus*.

Received: 29 January 2021
Accepted: 26 June 2023

Introduction

One of the gastrointestinal nematodes with the highest prevalence in ruminants in the world is *Haemonchus contortus*, which is considered the most pathogenic parasite of small ruminants (1). This blood-sucking nematode affects young and adult ruminants either clinically or sub-clinically, decreasing the efficiency of digestion (2, 3), reducing the energy metabolism of maintenance and production, causing anemia due to the severe loss of blood (4). Additional effects of this nematode are a reduction of feed intake, feed conversion, weight loss, and often high mortality in young animals (5), which causes significant economic losses (6, 7). Infections with *H. contortus* are ubiquitous in grazing goats in a wide range of ecosystems and induce subclinical and clinical diseases resulting in clinical diseases and productivity loss (8).

Currently, gastrointestinal parasites control in livestock mainly relies on repeated treatments with anthelmintic drugs such as benzimidazoles and macrocyclic lactones (9). However, multiple-drug anthelmintic resistance in gastrointestinal nematodes of livestock is highly prevalent throughout the world (10, 11) and anthelmintic resistance is a growing issue (12, 13), and new nematicides are not likely to offer long-lasting solutions because resistance

anthelmintic drugs can develop rapidly. Also, with the use of anthelmintic drugs, there is a risk of accumulation of residues of these drugs in meat and milk, and could damage other beneficial organisms such as manure beetles (*Onthophagus landolti*) (14). For this reason, it is desirable to use plant extracts that possess inhibitory effects against free-living helminths which can replace synthetic anthelmintic compounds.

Gliricidia sepium is an adaptable, fast-growing tree tropical legume distributed worldwide, due to its extensive introduction across tropical regions of the world. It has a lot of uses such as a fodder tree (15), fuelwood (16), living fences (17), and atmospheric nitrogen fixation (18). This tree is an important forage crop in cut-and-carry systems but its use has been limited by palatability (19) and low feed intake (20) due to various secondary metabolites (21).

G. sepium has been shown to possess ovicidal (22) and larvicidal (23) activity against *Haemonchus contortus*, a gastrointestinal nematode with high prevalence and resistant features in goats (24, 25). due to its wide array of secondary compounds. Alternative methods for control of gastrointestinal parasites are needed not only because of the development of anthelmintic resistance but also because of a demand by consumers for avoidance of chemicals and drugs in farm animals, and reduced chemical residues in the environment and milk and meat. This necessity is echoed in higher demand by consumers for organic and grass-fed livestock products (26).

Even though secondary compounds of *G. sepium* have demonstrated activity against gastrointestinal nematodes, additional information is required to attune the use of this plant as an anthelmintic alternative. Therefore, this study aimed to evaluate the in vitro nematicidal effect of hydro-methanolic extracts of *G. sepium* against *H. contortus*.

Materials and methods

Ethics statement

This study was conducted according to ethical principles and guidelines for experiments on animals of the Autonomous Agrarian University Antonio Narro (protocol number 3001-2258).

Plant material and hydro-alcoholic extract

Ten kg of *G. sepium* leaves (7 weeks regrowth; 2.5 m above the ground) were collected from 10 trees in a tropical zone of southwest Mexico (97°33´ W, 16°18´ N) at an altitude of 370 m above sea level. Mean annual temperature is 25°C and the average annual precipitation is 1409 mm. Partially dehydrated leaves of *G. sepium* were dried using a forced-draft forage dryer for 48 h at 70 °C. Samples were ground with a Wiley mill to a particle size <355 µm. One hundred g of the resulting plant material were added to a solvent containing 210 mL methanol and 90 mL water for 48 h at room temperature. The supernatant was sieved through No. 3 Whatman filter paper. The extract was placed in a rotary evaporator (Yamato RE300 rotavaporator) at 100 °C to get rid of methanol. The water fraction was extracted by a lyophilizer (Labconco, Kansas City, MO, USA) and the extract was placed in glass vials and stored at 4 °C until analysis. The extract was reconstituted to concentrations of 12.5, 25, 50, 100, and 200 mg/mL using distilled water.

Determination of condensed and hydrolyzable tannins

The HCL-Butanol technique (27) was used to obtain condensed tannins (CT). Three mL of HCL-terbutanol was added to 0.5 mL of the extract, in triplicate and 0.1 mL of ferric reagent (HCL-NH_4Fe $(SO_4)_2$) was added. This was done in test tubes with screw cap (13 × 10 mm), which were placed in a water bath at 100 °C for 1 h. Subsequently, they were allowed to cool to room temperature. The reading of tubes was carried out with a spectrophotometer (absorbance at 460 nm). The concentration was calculated using the catechin as standard and the results were expressed as mg/g in catechin equivalents (mg/CE/g DM).

Hydrolyzable tannins (HT) were determined using the Folin Ciocalteu technique (28). Forty µL of the diluted extract were used in 360 µL of distilled water, and this was deposited in 16 × 50 tubes for each of the extracts in triplicate. Subsequently, 400 µL of the Folin-Ciocalteu reagent (Sigma Aldrich F9252) was added, the mixture was homogenized and left to rest for 5 min, then 400 µL of sodium carbonate ($NaCO_3$, 0.01 M) were added. Mixtures were stirred and left to rest for 5 min. Finally, 2000 µL of distilled water was added and the absorbance of 725 nm was read with the spectrophotometer, the concentration was calculated using the gallic acid standard and the results were expressed as mg of gallic acid equivalent per g of DM of the plant extract (mg/GAE/g DM).

Partial purification of metabolites

An aqueous extraction was carried out as previously described for leaves of the trees for column chromatography (29), to partially purify compounds of the extractions. For this procedure, a vertical glass column with a capacity of 150 mL was used. The packing was carried out with Amberlite XAD-16 (Sigma Aldrich), 25 mL of the extract were deposited inside the adsorbent. The column was activated, step by step, with distilled water and MeOH: H_2O (40:60), and elution with water was carried out to remove sugars, amino acids, organic acids, and low molecular weight phenols.

Finally, the purified concentrate of metabolites was eluted with ethanol. The liquid obtained was fractionated in Petri dishes and placed in an oven at 60 °C for 24 h. The

metabolites were recovered as a fine powder and placed in vials (1.5 mL Eppendorf ™), protected from light.

For the identification of the secondary metabolites, 1 mg of leaves of *G. sepium* was weighed out of this fine powder and dissolved in 1 mL of methanol. Then the samples were subjected to sonic vibration for 20 min (Branson Sonicator model 2510). The samples were then filtered using membranes of 0.45 µm and placed in 1.8 mL glass vials and 0.75 mL of methanol were added to each vial, which was also filtered with 0.45 µm membranes to obtain a dilution of 1:2 of each sample in vials. The components of the plant were detected with the ProStar Varian HPLC system (Spectra Lab Scientific Inc., Markham, Ontario Canada), with a three-phase pump, a model 410 autosampler, and a diode array UV-vis detector. The column used for the analysis was a Varian Pursuit XRs c18, 4.6 mm x 250 mm, with a flow of 1 mL/min and a volume injection of 10 µL per sample. The mobile phases of analysis were: A methanol (washing phase), B acetonitrile, and C acetic acid 3%, with the following elution gradients 0-10 min 100% C, 10-20 min 20% B 80% C, 20 -25 min 30% B 70% C, 25-26 min 60% B 40% C, 26-31 min 30% B 70% C, 31-40 100% C (30). The column was washed and reconditioned for mass spectrophotometry analysis, using Varian 500/ MS equipment with ion trap, electrospray ionization (ESI), negative mode (MH-), capillary voltage of 90 V, and a mass range of 100-2000 m/z was used.

Larvae

Infective larvae of *H. contortus* were obtained from a previously worm-free 7-month-old lamb of approximately 30 kg which was infected orally with 350 *Haemonchus contortus* larvae (L3), per kg of live weight. The lamb was housed in a roofed pen and was fed a balanced commercial diet composed of concentrate and oat hay with free access to water. Coproparasitoscopic analyzes (McMaster chamber) were made weekly until day 21 post-infection when the parasite load was >800 eggs per g of feces. Feces were collected in plastic basins and were macerated. Then tap water was added to get a pasty consistency. Also, small polystyrene pellets were added to feces to enable homogenization and aeration of feces (31). The basin was sealed tightly to maintain a uniform relative humidity of 100% for 7 days to induce the hatching and exsheathing of larvae.

To get a suspension of larvae free of impurities, a filtering paper for cleaning the objectives of microscopes (Thomas Scientific, USA) was placed in a Baermann-funnel apparatus with tap water. Larvae descended using the optical lens paper and settled down on the bottom of the tube. The filtrated material was transferred into 50-mL plastic tubes and was kept at 4 °C for 1 h. Larvae were exposed to density gradients and centrifugation at 3500 rpm for 5 min. For this procedure, 4 mL of 40% sucrose was added to each of two 15-cm test tubes, then, with a Pasteur pipette, a pellet containing 2 mL of larvae was added slowly to the test tube and these tubes were centrifuged at 3500 rpm for 5 min. This process decanted fecal debris at the bottom of the tube. After centrifugation, a package of concentrated clean larvae (white ring) appeared on the surface of the suspension. These were collected and deposited in 10 mL tubes where they were washed three times with distilled water. This eliminated excess sucrose and maintained an appropriate osmotic balance for the survival of larvae. Cleaned larvae were placed in 30 mL cell cultures with 10 mL of clean water and were kept at 4 °C until quantification. Eleven 10 µL aliquots were used to count larvae on a slide under a microscope with a × 4 magnification. Larvae were placed in culture plates and kept at 4 °C. Sodium hypochlorite (0.186 µL) was used to remove the sheath of the infective larvae, and when larvae were observed to leave the sheath, they were washed with purified water to remove sodium hypochlorite.

Larval survival assay

The in vitro bioassays comparison (extract-larvae) was carried out on 96-well micro-titration plates. For each well, 50 µL of the extract was mixed with 12.5, 25, 50, 100, and 200, mg/mL with three replicates for each concentration and controls and in triplicate. A total of 110 larvae (either sheathed or exsheathed) were seeded into each well containing the extract. Then the plate was protected with a soaked paper towel to maintain moisture and then enclosed with aluminum foil. Survival determinations were made at 24, 48, and 72 h. For determination of survival, 11 10 µL aliquots were positioned on a slide and observed in a microscope with a × 10 magnification. The plates were incubated at 20 °C and 80% relative humidity for 24, 48, and 72 h in an oven. For the positive control, 50 µL of 1% ivermectin (Aranda Laboratories, Queretaro, Mexico) and 110 infective larvae contained in 50 µL per well were used.

The mortality of larvae at different extract concentrations was evaluated, and a percentage of larval mortality was calculated. After putting *H. contortus* in contact with the aqueous extract, motility was observed every 6 h using a magnifying glass. Adult worms' motility inhibition was evaluated as the following ratio: the number of immotile (dead) nematodes divided by the total number of initially mobile (alive) nematodes for each concentration or control. The death of larvae of *H. contortus* was determined by the lack of motility for five seconds.

Statistical analyses

The effect of concentration of hydro-methanolic extracts of *G. sepium* on larvae of *H. contortus* survival was analyzed using the GENMOD procedure of SAS (SAS Institute, Inc., Cary, NC, USA). The experimental units were the groups of larvae. For sheathed or exsheathed larvae the model included the effect of levels of extract, days of incubation, and simple interaction. For larvae survival, time of incubation

and the interaction treatment × time of incubation were non-significant (P>0.15) and thus, were excluded from the model, and data were pooled across extract levels. Differences among levels of extract were determined by the LSMEANS/DIFF option of SAS. The median lethal concentration (LC50) was analyzed by the probit method (PROC PROBIT of SAS). Models for the associations between extract level and larvae mortality were described using the CurveExpert software (CurveExpert Professional 2.5.6; Hyams Development, Huntsville, Alabama). P-values less than 0.05 were considered statistically significant.

Results

After lyophilization, the hydro-methanolic extract gave 6.8 g from 100 g of leaves of *G. sepium*. Total, condensed and hydrolyzable phenolic compounds obtained from leaves of *G. sepium* were 6.4 ± 2.4, 3.4 ± 1.2, and 3.1 ± 1.7 mg/g of dry matter, respectively (means ± SD). The compounds distinguished in the extracts from leaves of *G. sepium* by HPLC-mass spectrometry are presented in table 1. Proanthocyanidin and phenolic acid were found, as well as various flavonols and flavonoids.

The extracts of *G. sepium* exhibited a significant (P<0.001) sheated larvae mortality at concentrations of 12.5 and 25 mg/mL at 24 and 72 h post-incubation compared with the negative control (figure 1). Extract of *G. sepium* at concentrations of 50 and 100 mg/mL did not differ (P>0.05) between them in mortality rate of larvae but they were less effective (P<0.05) than lower extract concentrations. The larval mortality assay had a cubic dose-related mortality response for the sheathed larvae (Fig. 1) with 61.5 ± 20.4% mortality rate with the highest concentration (200 mg/mL) after 72 h of incubation.

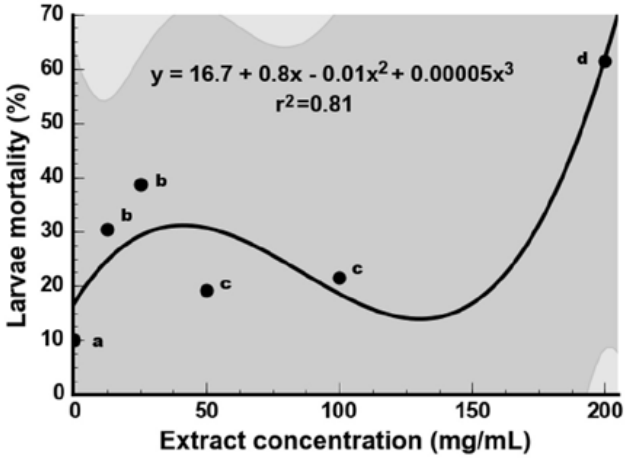

Figure 1: Sheathed larvae of *Haemonchus contortus* mortality assay concentration–response curve for hydro-methanolic extracts of *Gliricidia sepium*. Ivermectin was the positive control leading to 100% inhibition at concentration of 10 mg/mL used. The negative control consisting of plain water (value at 0 mg/mL extract concentration in the X axis) led to about 10% inhibition. Darker bands are 95% confidence intervals for predicted values. Lighter bands are 95% confidence intervals for actual values. Extract concentrations with different letters differ (*P*<0.05)

Regarding the exsheathed larvae, an extract concentration of 12.5 mg/mL did not alter larvae survival after incubation for 72 h (figure 2). Mortality rate of exsheathed larvae was less than 25% for 72 h incubation using 25, 50, and 100 mg/mL concentrations. The larval mortality assay had a quadratic dose-related mortality response in the sheathed larvae (Fig. 2) with 93.8 ± 2.9% mortality for a dilution of 200 mg/mL and incubation of 72 h.

Viability assay LC50 of *G. sepium* hydro-methanolic extract for sheathed and exsheathed larvae is shown in table 2. *G. sepium* had the highest LC50 (lowest toxicity) of 40 and 68 mg/mL for 48 and 72 h of incubation of exsheathed larvae

Table 1: Compounds identified by HPLC-mass spectrometry in the extracts of leaves of *Gliricidia sepium*

Retention time (min)	Mass/charge	Compound	Family
16.5	233	Vanillin 4-sulfate	Flavonoids
28.7	885	Prodelphinidin	Proanthocyanidins
29.6	325	p-Coumaroyl glucose	Phenolic acid
30.7	755	Kaempferol 3-o-glucosyl-rhamnosyl-glucoside	Flavonols
31.8	739	Kaempferol-3-O-xylosyl rutinoside	Flavonols
33.2	165	p-Coumaric acid	Phenolic acid
34	593	Luteolin 7-rutinoside	Flavonoids
35	623	Isorhamnetin 3-glucoside-7-rhamnoside	Flavonols
37	385	Dihydroferulic acid	Phenolic acid

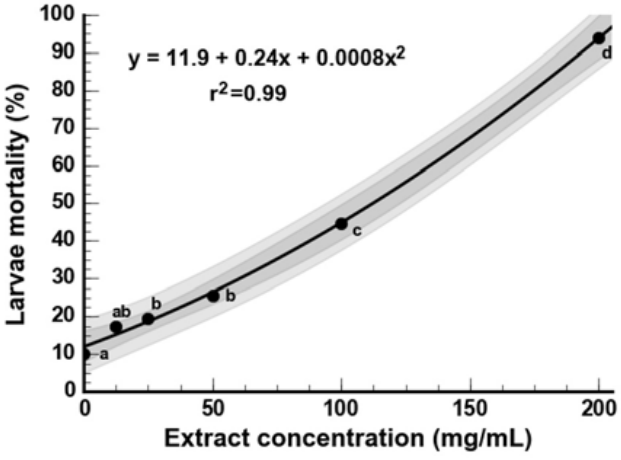

Figure 2: Exsheathed infective larvae of *Haemonchus contortus* mortality assay for different concentrations of hydro-methanolic extracts of *Gliricidia sepium*. Ivermectin was the positive control leading to 100% inhibition at concentration of 10 mg/mL used. The negative control consisting of plain water led to about 10% inhibition. Darker bands are 95% confidence intervals for predicted values. Lighter bands are 95% confidence intervals for actual values. Extract concentrations with different letters differ (P<0.05)

of *H. contortus*. Observations of the sheathed infective larvae of *H. contortus* subjected to the hydro-methanolic extract of *G. sepium* showed the presence of lesions on the nematode cuticle which were similar to those provoked by 1% ivermectin.

Discussion

In this study, extracts of leaves of *G. sepium* using hydro-methanolic solvents were examined for their anthelmintic activities. The phytochemical study showed that *G. sepium* was rich in flavonoids, flavonols but not total polyphenols. The phenolic contents of the analyzed extracts were similar to those previously reported (32). Also, the results of this study are consistent with previous results from other studies, which confirmed the high concentration of flavonoids and phenolic acid detected in *G. sepium* associated with anthelmintic activity in *G. sepium* (21).

In the present study, the in vitro model showed that hydro-methanolic extract from *G. sepium* had a dose-dependent effect on the sheathed and exsheated larvae of *H. contortus*, with the highest larvae mortality at 200 mg/mL. This concentration is much higher than the 1.25 mg/mL acetonic extract of *G. sepium* for eliminating >92% of L1 and L2 larvae of this blood-sucking nematode (33). Also, it was observed (34) that the percentage of exsheathment of *H. contortus* with 1200 µg/mL of *G. sepium* hydro-acetone extracts was 71.3%. A much lower concentration of ethanol extract of G. sepiun (40 mg/mL) than those used in the present study was adequate for in vitro larval exsheathment inhibition of *G. sepium* (21).

Efficiency of anthelmintic drugs adopted by the W.A.A.V.P. should inhibit larval motility by more than 90% (35). Thus, these in vitro results obtained with *G. sepium* extract against exsheathed infective larvae of *H. contortus* at the highest concentration, would classify the tested extract as effective. In the case of sheathed larvae of *H. contortus* the hydro-methanolic extract of *G. sepium* can be considered moderately effective against this nematode. It should be noted that the hydro-methanolic extract of *G. sepium* at its highest concentration exhibited an anthelmintic efficiency against exsheathed infective larvae of *H. contortus* close to that observed for the positive control (99.3%) in the nematode motility test. This result is important because it would reduce the chances of developing resistance of the exsheathed infective larvae of this nematode to the extract of *G. sepium* (36).

The mortality of exsheated and sheated larvae of *H. contortus* increased as the exposure period of the *G. sepium* extract went from 24 to 72 h. So, the nematode toxicity to *G. sepium* extract depends not only on the concentration of extracts but also on the exposure period. This is in line with previous studies in vitro where mortality rates of larvae of *H. contortus* increased with the increase in exposure time to plant extracts (37).

This leguminous tree undoubtedly contains nematotoxic constituents that cause the death of most of the exsheated larvae of this nematode. The chemicals contained in *G. sepium* either in a single form or in a combination induced mainly the death of exsheated larvae of *H. contortus*, which is in line with earlier studies that have shown that hydro-acetone and ethanolic extracts of this leguminous tree have ovicidal (21,22) and larval exsheathment inhibition (32).

It has been indirectly demonstrated (32) that tannins/polyphenolic compounds of *G. sepium* were involved in the anthelminthic activity of leaves from this tree. However, in the present study total polyphenols in the leaves of this legume were low (6.4 mg/g), thus other secondary metabolites of this leguminous tree other than polyphenols seem to be

Table 2: Viability assay (LC50) of *Gliricidia sepium* hydro-methanolic extract for sheathed and exsheathed larvae of *Haemonchus contortus*. Values are mg/g

Larvae	24 h	CI*	48 h	CI	72 h	CI
Sheathed	41	10-88	70	33-100	74	46-100
Exsheathed	23	1-81	40	8-94	68	32-100

*CI confidence interval

involved in the mortality of sheated and exsheated larvae of *H. contortus*. Flavonols contained in leaves of *G. sepium* could be involved in the death of this nematode because these chemical compounds have shown antimicrobial activity largely due to the ability of these compounds to hamper the cytoplasmic membrane function (38) and the nematicidal activity against *H. contortus* of flavonoids alone (39, 40) or in combination with condensed tannins (41) has been documented.

After exposure of sheated and exsheated larvae of *H. contortus* to extract of *G. sepium*, damages on the cuticle of this nematode were similar to those produced by ivermectin. Thus, the antiparasitic activity of the secondary compounds of *G. sepium* in larvae seems to be explained by their contact with the nematode's cuticle. However, visualization of the structural changes in the nematode by electron microscopy would be necessary to confirm this damage in this gastrointestinal parasite.

It is concluded that extract of leaves of *Gliricidia sepium* showed significant dose-dependent mortality of sheated and exsheated larvae of *H. contortus*. Flavonoids, flavonols, phenolic acid, and tannins apparently were responsible for the anthelmintic bioactivity of this plant. Since *G. sepium* is an important cultivated plant in tropical regions and toxicity has not been demonstrated for livestock, the leaves extract of this leguminous tree possesses in vitro larvicidal activity against *H. contortus*. However, purification will be necessary to isolate and purify the bioactive compounds from *G. sepium* extracts, to further evaluate their in vivo activities on gastrointestinal parasites, and to study the structural changes in the *H. contortus* by electron microscopy to determine the mode of action of the secondary compounds of *G. sepium* on this blood-sucking nematode.

Acknowledgements

The authors are thankful to the personnel of the Laboratory of Helminthology (CENID-PAVET, National Institute for Research in Forestry, Agriculture and Animal Production, Cuernavaca, Mexico) for allowing us to carry out part of this experiment in their facilities.

Conflict of interests. All authors declare that there are no actual or potential conflicts of interest between the authors and other people or organizations that could inappropriately bias their work.

Founding. Autonomous Agrarian University Antonio Narro (grant 3001-2258).

Author contributions. MM and JEG designed and drafted the manuscript. UMC carried out the statistical analysis. LG carried out the field and laboratory work. LAR and UMC revised the manuscript and reviewed the pertinent literature. Finally, all authors revised the manuscript and approved the submitted version.

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Učinki hidrometanolnega izvlečka listov *Gliricidia sepium* na ličinke *Haemonchus contortus in vitro*

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Izvleček: Namen te *in vitro* študije je bil oceniti protiglivične učinke izvlečkov *Gliricidia sepium* na ličinke *Haemonchus contortus* z ovojem in brez njega. Ličinke parazita so bile inkubirane 24, 48 ali 72 ur pri 20–25 °C v hidrometanolnih izvlečkih listov tega tropskega drevesa v koncentracijah 12,5, 25, 50, 100 in 200 mg/ml. Voda in ivermectin sta služila kot negativna in pozitivna kontrola. Skupne fenolne spojine v listih *G. sepium* so obsegale 6,4 ± 2,4 mg/g suhe snovi. Druge spojine, ki so bile v drevesu identificirane s HPLC-masno spektrometrijo in ki bi lahko bile odgovorne za opažene protiglivične učinke, so bile vanilin 4-sulfat, prodelfinidin p-kumaroil glukoza, kaempferol 3-O-glukozil-rimnozil-glukozid, kaempferol-3-O-ksilozil rutinozid, p-kumarna kislina, luteolin 7-rutinozid, izorhamnetin 3-glukozid-7-rimnozid in dihidro ferulinska kislina. Pri odmerkih 100 mg/ml in 72 urah inkubacije je bila stopnja smrtnosti pri *H. contortus* z ovojem 21,6 %, pri *H. contortus* brez ovoja pa 44,7 %. Pri odmerkih 200 mg/ml in 72 urah inkubacije so hidrometanolni izvlečki *G. sepium* uničili 61,5 % ličink z ovojem in 93,8 % ličink brez ovoja. Srednja efektivna koncentracija (EC50) izvlečka *G. sepium* za ličinke z ovojem je bila 74 mg/ml (CI = 46–100), za ličinke brez ovoja pa 68 mg/ml (CI = 32–100) po 72 urah inkubacije. Statistično značilna ($P < 0,001$) sposobnost uničenja ličink v primerjavi z negativno kontrolo kaže na protiglivične lastnosti *G. sepium* proti *H. contortus in vitro*.

Ključne besede: flavonoidi; flavonoli; ličinke; nematodi; tanini

DOI 10.26873/SVR-1564-2023
UDC 636.92:591.53:577:591.4:616.15
Pages: 135–47

Original Research Article

Influence of Feed Restriction and Zinc Oxide Nanoparticles Supplementation on Growth Performance, Blood Biochemistry, Intestinal Morphology and Cecal Fermentation Parameters of Growing Rabbits

Key words

feed restriction;
growth;
rabbits;
Zn oxide nanoparticles

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Abstract: The present study investigated the response of growing rabbits in terms of growth performance, serum biochemical, intestinal morphology, and caecal fermentation parameters to feed restriction and zinc oxide nanoparticles (ZnO-NPs) supplementation. A total of 60 New Zealand male rabbits were randomly distributed into 6 groups: AL-0 (fed *ad libitum* + fresh water as control); AL-15 and AL-30 (*ad libitum* + water supplemented with ZnO-NPs in water, 15 and 30 mg/L, respectively); and R-0, R-15 and R-30 were the same as the first 3 groups but with restricted feeding regime. Rabbits fed *ad libitum* and supplemented with ZnO-NPs (15 mg/L) showed the highest body weight with no significant difference from AL- fed groups or R-0. Feed conversion ratio (FCR) showed no difference among the different experimental groups ($P > 0.05$). ZnO-NPs supplementation reduced the serum lipid profile parameters, catalase enzyme in R-30, superoxide dismutase in AL-15 and AL-30 while increased serum malondialdehyde (MDA) in both *ad libitum* and restricted rabbits. ZnO-NPs administration resulted in lower caecal ammonia in AL-30 compared to its control (AL-0) as well as the content of individual volatile fatty acids (VFAs) (acetate, butyrate and propionate) ($P < 0.05$). Ileum morphological parameters (mucosal length, villi length, and goblet cell number) were modified in response to the feed restriction and ZnO-NPs addition. In conclusion, feed restriction program applied in this experiment altered rabbit growth performance (final body weight and weight gain with no differences in FCR), improved ileum morphology while had no significant effect on caecal fermentation (VFAs profile) or microbiological parameters. ZnO-NPs supplementation in both levels (15 and 30 mg/L) differently modulated serum lipid profile, antioxidant enzymes and MDA, VFAs profile in cecum and ileal morphology with no differences in rabbit growth performance.

Received: 3 September 2022
Accepted: 30 May 2023

Introduction

In mammals, the peri-weaning period is considered one of the main stress periods in animal life as the young animal shifts from mother's milk to the solid feed. During this critical period, fast growth occurs accompanied by many problems such as high incidence of metabolic disorders, high morbidity and mortality, and digestive disturbances, which have deleterious effects (1), causing economic losses in the commercial rabbit production. Antibiotics have been used to control these disturbances, but the emergence of

antibiotic resistance has heightened the need to find other solutions to this problem. Several approaches have been considered to protect rabbit health during this stressful period, such as nutritional modulation and hygienic control. One of these approaches is the feed restriction, which can be done in two ways: quantitatively or qualitatively. Qualitative restriction involves limiting the amount of nutrients like protein and energy in feed (2). The quantitative restriction of feed can be done by limiting the feeder access time or reducing the amount of offered feed (2). Previous trials informed beneficial effects of feed restriction as it stimulated compensatory growth, improved feed efficiency utilization (3), improved digestibility of nutrients, lowered fat accumulation in carcasses (4, 5), and reduced the post-weaning digestive disturbance as the epizootic rabbit enteropathy (6). Beside these beneficial effects, also some negative effects such as reduced final weight and dressing out percentage in feed restricted rabbits, were reported (7, 8).

Another different approach is through the supplementation of additives in feed or water. Zinc (Zn) is an essential trace element which plays a vital role in cell division, synthesis, and stabilization of DNA (9). Moreover, it has many beneficial effects on different physiological functions such as acid-base balance, nutrient metabolism, and immune response (10, 11). MacDonald (12) reported that Zn improved feed utilization through participating in the metabolism and assimilation of different nutrients such as carbohydrates, proteins, and lipids. Recently, manufactured nanoparticles (NP) have shown new characteristics such as great specific surface area, high surface activity, a lot of surface-active centers, and high catalytic efficiency (13). Due to the advantage of small size and high surface reactivity, nanoparticles showed higher transport, uptake and increased absorption efficiencies, enhancing their bioavailability inside the animal body (14). The inclusion of nano additives especially nano minerals in animal nutrition has been widely adopted to enhance the growth and production of livestock. Zinc nanoparticle supplementation was found to be beneficial in rabbits (15, 16), piglets (17) and poultry (18, 19). Therefore, the aim of the present study was to investigate the effect of feed restriction and ZnO-NPs supplementation in the drinking water on the growth performance, some blood biochemical parameters, intestinal morphology, caecal microbiology, and volatile fatty acids fermentation in growing rabbits.

Materials and methods

Ethical statement

Animal management procedures were undertaken in accordance with the requirements of the Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, Alexandria University, Egypt (AU 013-2022/10/12-3-149).

Rabbit care and experimental design

Sixty New Zealand male weaned rabbits; 33-35 days old (average body weight 770 ± 5.96 g) were randomly distributed into 6 experimental groups (10 rabbits/group) with 3 replicates/group (3-4 rabbits/each). Experimental groups were arranged as follows: AL-0 (rabbits were fed *ad libitum* + fresh water as control); AL-15 and AL-30 (rabbits fed *ad libitum* + water supplemented with ZnO-NPs, 15 and 30 mg/L respectively); R-0 (restricted feed + fresh water); R-15 and R-30 (restricted feed + water supplemented with ZnO-NPs, 15 and 30 mg/L respectively). The restriction program applied throughout a 2-month experimental period was done as rabbits were fed *ad libitum* (AL) at the first day, next day they were fed at a level of 93% of AL, then again AL and next day 93% of AL of the day before (20). The zinc oxide water dispersion nanoparticles used were <100 nm particle size (TEM), (Sigma-Aldrich, catalog No.721077). Growing rabbits were kept on a commercial pelleted diet illustrated in table 1. The commercial diet used was formulated to meet the nutrient requirements of the growing rabbits according to (21). Rabbits were kept in wire-galvanized batteries that had feeders and drinkers. All the animals were kept under the same management and hygienic conditions.

Growth performance: To implement the feed restriction program and ZnO-NPs supplementation, rabbits were weighed every 2 weeks, and feed intake (FI) was measured daily for all the experimental groups. Feed conversion ratio (FCR) and weight gain (WG) were calculated in accordance.

Sample collection: At the end of the experiment, three rabbits from each group were chosen at random and used for sample collection. Blood was drawn from the rabbit ear vein for serum separation and biochemical parameter analysis. Rabbits were then sacrificed with an overdose of pentobarbital sodium at 60 to 70 mg/kg live weight. The ileum specimen was preserved in 10% formalin for histopathological examination. Caecal content (one gram) was collected under aseptic conditions for microbiological examination, while the remainder was used for VFAs fermentation analysis.

Blood biochemical parameters: Blood samples were collected in clean vials without anticoagulant. The serum was separated by centrifugation at 3000 rpm for 10 min. Samples were used for the analysis of some serum biochemical parameters including total protein, albumin, glucose, triglycerides (TG), total cholesterol (TC), low and high-density lipoproteins (LDL and HDL), some liver function enzymes as aspartate amino transferase (AST) and alanine amino transferase (ALT), some kidney function parameters (uric acid and creatinine), some antioxidant enzymes as superoxide dismutase (SOD) and catalase, serum zinc concentration, and malondialdehyde (MDA) using commercial kits produced by Bio-diagnostic Co. (Diagnostic and Research reagents).

Intestinal morphology: The fixed ileal tissue was embedded in paraffin blocks, sectioned (5 µm), stained with haematoxylin and eosin (H&E) as previously described by Bancroft et al. (22), and then examined using a light microscope. The morphometric measurement of various parameters of intestinal villi and their associated crypt was performed quantitatively using image J software (Bethesda, MD, USA) according to Abràmoff et al. (23).

Table 1: Diet ingredient composition used during the experiment

Ingredients	**g/kg**
Berseem hay	305
Yellow corn	90
Barely	203.5
Wheat bran	180
Soybean Meal (44%)	160
Wheat straw	20
Molasses	20
Dicalcium phosphate	8
Calcium carbonate	7
Common Salt	3
Mineral and vitamin premix[1]	3
Dl-methionine	0.5
Chemical composition (%)	
Dry matter	87.32
Crude protein	17.01
Ether extract	2.60
Crude fiber	12.18
Nitrogen free extract	49.02
Ash	6.51
Digestible energy (DE) MJ/kg diet	10.46

[1]Mineral and vitamin premix composition (per 3kg): Vitamin A = 12,000,000 IU, D3 = 2,000,000 IU, E = 10,000 mg, K3 = 1,000 mg, B1 = 1,000 mg, B2 = 5,000 mg, B6 = 1,500 mg, B12 = 10 mg, Niacin = 30,000 mg, Biotin = 50 mg, Folic acid = 1,000 mg, Pantothenic acid = 10,000 mg, Choline chloride = 500,000 mg, Zinc = 50,000 mg, Manganese = 60,000 mg, Iron = 30,000 mg, Copper = 10,000 mg, Iodine = 1,000 mg, Selenium = 100 mg, Cobalt = 100 mg, Calcium carbonate to 3kg

Caecal microbiology and volatile fatty acids: According to Mountzouris et al. (24), the collected cecal contents were serially diluted ten times. The total bacterial and total coliform counts were calculated using the method described by Bivolarski et al. (25). The remaining cecal contents were immediately collected, squeezed with sterile gauze to obtain the caecal filtrate, and the residues were discarded. The caecal filtrate was diluted with an equal volume of diluted sulfuric acid before the pH was determined (using a digital pH meter). The prepared filtrate samples were frozen at -20°C for subsequent analysis of ammonia and VFAs concentrations according to the methods described by Alvarenga et al. (26).

Statistical analysis: The obtained data were subjected to two-way ANOVA to test the effects of different levels of ZnO-NPs, feed restriction as well as their interaction. Statistical analysis was conducted using GraphPad Prism 6 (GraphPrism Software, La Jolla, CA, USA). The obtained data were presented as mean ± standard error (SE) and significance was considered at P< 0.05.

Results

Growth performance of growing rabbits

Table 2 illustrates the rabbit's growth performance in response to ZnO-NPs supplementation and feed restriction. Applying the feed restriction significantly affected the rabbit's growth performance in terms of final body weight (BW), weight gain (WG), and feed intake (FI) ($P < 0.05$). The final BW and WG of rabbits under restriction and supplemented with ZnO-NPs (15 mg/L) were significantly reduced ($P < 0.05$) compared with the same group of rabbits freely fed and receiving the same dose of ZnO-NPs. The ZnO-NPs administration in the water had no significant effect ($P > 0.05$) on BW or WG either in the *ad libitum* or restricted rabbits. However, rabbits fed *ad libitum* and supplemented with ZnO-NPs (15 mg/L) showed the highest BW and WG ($P > 0.05$).

A non-significant reduction of FI ($P > 0.05$) was obtained in restricted rabbits compared with their relative control rabbits. The effect of the restriction regime was clear in those supplemented with ZnO-NPs (30 mg/L) as FI was significantly reduced ($P < 0.05$) compared with their control group, which received the same dose but fed freely. Neither feed restriction nor Zn supplementation affected the FCR ($P > 0.05$) of growing rabbits, although it was non-significantly improved in *ad libitum* fed rabbits supplemented with the lower dose of ZnO-NPs (15 mg/L).

Blood biochemical parameters

Blood serum concentrations of total protein and glucose showed no difference ($P > 0.05$) among different experimental groups; however, glucose concentration

Table 2: Effect of feed restriction and Zinc nanoparticles (ZnO-NPs) supplementation on rabbit growth performance

ZnO-NPs (mg/L)		Initial body weight (g/ rabbit)	Final body weight (g/ rabbit)	Total weight gain (g/ rabbit)	Feed intake (g/ rabbit)	Feed conversion ratio
AL-0	*Ad libitum*	774.50±14.92	2142.50±88.40^{AB}	1368.00±83.98^{AB}	7148.29±53.10^{A}	5.23±0.36
AL-15		763.50±16.33	2251.00±44.90^{A}	1487.50±39.99^{A}	7104.00±60.58^{AB}	4.77±0.10
AL-30		766.50±17.51	2065.56±71.18^{AB}	1299.06±73.12^{AB}	7239.85±190.42^{A}	5.57±0.36
R-0	Restricted	776.50±17.65	2077.50±69.99^{AB}	1301.00±72.38^{AB}	6924.10±212.14^{AB}	5.32±0.39
R-15		775.00±14.26	2042.50±105.37^{B}	1257.50±110.61^{B}	6970.32±247.79^{AB}	5.54±0.39
R-30		764.00±17.43	1971.50±21.63^{B}	1207.50±25.82^{B}	6697.78±108.04^{B}	5.54±0.16
Two-way Anova (*P-value*)						
Feed offering regime		0.785	0.039	0.031	0.026	0.230
ZnO-NPs level		0.821	0.187	0.246	0.886	0.544
Interaction		0.909	0.573	0.482	0.414	0.164

Values shown in table are mean ± SE. Uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups

was numerically increased in feed restricted rabbits irrespective of ZnO-NPs supplementation (Table 3). The interaction between the feed restriction regime and ZnO-NPs supplementation significantly altered the serum albumin and globulin concentrations ($P < 0.05$). Albumin concentration was significantly reduced in *ad libitum*-fed rabbits supplemented with 15 mg/L of ZnO-NPs, while showed no differences ($P > 0.05$) in the restricted ones.

Reducing the amount of feed significantly increased ($P < 0.05$) the globulin concentration when compared with rabbits fed freely without added ZnO-NPs (control). Unlike albumin, serum globulin concentration was significantly increased in full-fed rabbits supplemented with 15 mg/L of ZnO-NPs, while showed no differences ($P > 0.05$) in the restricted ones.

Table 3: Effect of feed restriction and Zinc nanoparticles (ZnO-NPs) supplementation on some blood serum biochemical parameters of growing rabbits

ZnO-NPs (mg/L)		Total Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Albumin/ globulin ratio	Glucose (mg/dL)
AL-0	*Ad libitum*	5.96±0.05	5.20±0.09^{aA}	0.76±0.14^{bB}	7.49±1.77	93.07±2.41
AL-15		6.01±0.04	4.85±0.11^{bB}	1.16±0.14^{aA}	4.30±0.56	99.56±1.14
AL-30		6.03±0.02	4.97±0.03^{abAB}	1.06±0.05^{aA}	4.69±0.25	102.90±5.12
R-0	Restricted	6.01±0.03	4.97±0.12^{aAB}	1.04±0.09^{aA}	4.84±0.49	104.36±6.38
R-15		6.02±0.01	5.12±0.01^{aA}	0.90±0.00^{aAB}	5.71±0.02	107.35±4.39
R-30		6.02±0.04	5.08±0.02^{aAB}	0.94±0.04^{aAB}	5.41±0.25	106.06±3.77
Two-way Anova (*P-value*)						
Feed offering regime		0.538	0.422	0.654	0.795	0.053
ZnO-NPs level		0.444	0.482	0.367	0.292	0.378
Interaction		0.667	0.019	0.029	0.055	0.639

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups

Table 4: Effect of feed restriction and Zinc nanoparticles (ZnO-NPs) supplementation on the serum concentrations of antioxidant enzymes (catalase, superoxide dismutase), malondialdehyde and serum zinc concentration in growing rabbits

ZnO-NPs (mg/L)		Catalase (U/ml)	SOD (U/ml)	MDA (nmol/ml)	Zinc (mg/dL)
AL-0	*Ad libitum*	145.16±8.69^{aA}	427.67±36.67^{a}	9.56±0.40^{b}	3.59±0.22^{bB}
AL-15		124.37±12.15^{aAB}	304.37±12.40^{b}	12.69±0.38^{a}	5.11±0.14^{aA}
AL-30		107.52±1.50^{aA}	292.60±9.05^{b}	14.37±0.81^{a}	4.81±0.32^{abAB}
R-0	Restricted	89.95±6.97^{aB}	346.31±38.28^{a}	10.66±0.36^{b}	3.94±0.16^{bB}
R-15		120.92±4.98^{aAB}	314.10±14.64^{a}	12.98±0.52^{a}	4.28±0.20^{bAB}
R-30		42.24±18.32^{bB}	274.00±19.21^{a}	13.75±0.24^{a}	5.43±0.22^{aA}
Two-way Anova (*P-value*)					
Feed offering regime		0.0004	0.160	0.519	0.781
ZnO-NPs level		0.001	0.003	<0.0001	0.0001
Interaction		0.023	0.207	0.247	0.013

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups.

Table 4 shows the effects of experimental treatments on the serum concentrations of antioxidant enzymes (SOD and catalase), MDA, and serum Zn concentration. Catalase enzyme activity was significantly altered with ZnO-NPs addition, feed restriction applied, and the interaction between them ($P < 0.05$). Lower enzyme activity was observed in restricted rabbits compared with those freely fed. With ZnO-NPs supplementation, reduced catalase activity was found in restricted rabbits received 30 mg/L. SOD enzyme activity nearly followed the same trend of reduction which

Table 5: Effect of feed restriction and Zinc nanoparticles (ZnO-NPs) supplementation on some serum liver and kidney function related parameters of growing rabbits

ZnO-NPs (mg/L)		AST[1] (U/L)	ALT[2] (U/L)	Uric acid (mg/dL)	Creatinine (mg/dL)
AL-0	*Ad libitum*	38.33±2.60^{aB}	27.00±4.00	6.17±0.03	1.97±0.04
AL-15		43.00±1.00^{aAB}	31.00±4.58	6.08±0.07	1.95±0.04
AL-30		40.67±1.45^{aAB}	29.00±1.73	6.09±0.05	1.91±0.04
R-0	Restricted	47.33±3.84^{aA}	32.67±2.03	6.14±0.02	1.98±0.01
R-15		37.67±0.88^{bB}	32.00±1.73	6.00±0.06	1.98±0.04
R-30		39.00±1.73^{bB}	27.00±1.53	6.14±0.02	1.94±0.06
Two-way Anova (*P-value*)					
Feed offering regime		0.714	0.519	0.625	0.511
ZnO-NPs level		0.367	0.496	0.084	0.437
Interaction		0.017	0.430	0.396	0.920

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups. [1]AST: aspartate amino transferase, [2]ALT alanine amino transferase.

Table 6: Effect of feed restriction and Zinc Nanoparticles supplementation (ZnO-NPs) on serum lipid profile of growing rabbit

ZnO-NPs (mg/L)		Cholesterol (mg/dL)	Triglyceride (mg/dL)	HDL[1] (mg/dL)	LDL[2] (mg/dL)	VLDL[3] (mg/dL)
AL-0	*Ad libitum*	86.63±3.50^{aA}	110.03±1.92^{a}	21.37±0.77^{a}	43.26±2.36^{a}	22.01±0.38^{a}
AL-15		70.87±1.86^{bAB}	92.97±1.65^{b}	17.13±0.38^{b}	35.14±2.26^{b}	18.59±0.33^{b}
AL-30		72.77±2.10^{bAB}	96.10±4.37^{b}	19.30±0.93^{ab}	34.25±3.66^{b}	19.22±0.87^{b}
R-0	Restricted	80.70±1.81^{aA}	108.33±2.19^{a}	20.23±0.3^{a}	38.80±2.45^{a}	21.67±0.44^{a}
R-15		78.27±1.40^{abAB}	99.00±1.74^{ab}	18.57±0.70^{a}	39.90±1.40^{a}	19.80±0.35^{b}
R-30		69.50±1.92^{bB}	89.97±2.64^{b}	17.00±0.86^{b}	34.51±1.24^{a}	17.99±0.53^{c}
Two-way Anova (*P-value*)						
Feed offering regime		0.736	0.781	0.263	0.092	0.781
ZnO-NPs level		0.0002	<0.0001	0.002	0.048	<0.0001
Interaction		0.020	0.098	0.054	0.192	0.098

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between ad libitum and restricted fed groups. [1]HDL: high density lipoprotein, [2]LDL: low density lipoprotein, [3]VLDL: very low- density lipoprotein

was observed also with increasing ZnO-NPs level in the water especially in the *ad libitum* rabbits ($P < 0.05$). MDA as an important indicator of oxidative stress, was significantly increased with ZnO-NPs supplementation (P< 0.05) compared to rabbits received fresh water without Zn. ZnO-NPs supplementation altered the serum Zn concentration, which was significantly increased ($P < 0.05$) in either *ad libitum* or restricted rabbits. The highest serum Zn concentration was shown in the 15 mg/L ZnO-NPs supplemented *ad libitum* rabbits, and the 30 mg/L restricted fed rabbits.

As presented in table 5, kidney function-related parameters such as uric acid and creatinine were not affected by the feed offering regime or ZnO-NPs administration or the interaction

Table 7: Effect of feed restriction and Zinc Nanoparticles (ZnO-NPs) supplementation on volatile fatty acids in caecum of growing rabbits

ZnO-NPs (mg/L)		Ammonia (mg/dL)	Total volatile fatty acids (mM)	Acetate (% of total VFAs)	Propionate (% of total VFAs)	Butyrate (% of total VFAs)
AL-0	*Ad libitum*	14.90±0.38^{a}	50.40±3.72	71.73±1.16^{aA}	6.03±0.33^{a}	24.23±1.19^{a}
AL-15		13.00±0.42^{ab}	45.67±1.17	63.47±0.81^{bAB}	4.80±0.35^{b}	20.80±0.80^{b}
AL-30		12.20±0.29^{b}	40.67±6.23	63.80±1.00^{bAB}	4.90±0.42^{b}	19.17±0.73^{b}
R-0	Restricted	13.67±0.64^{a}	49.93±3.44	67.07±0.86^{aAB}	5.33±0.03^{a}	22.57±0.55^{a}
R-15		13.23±0.24^{a}	40.50±4.65	67.27±0.80^{aAB}	5.03±0.24^{a}	23.07±1.23^{a}
R-30		12.30±0.74^{a}	41.90±1.05	62.90±1.50^{bB}	4.93±0.20^{a}	20.03±0.41^{b}
Two-way Anova (*P-value*)						
Feed offering regime		0.462	0.613	0.505	0.552	0.505
ZnO-NPs level		0.004	0.077	0.0003	0.031	0.003
Interaction		0.284	0.962	0.006	0.274	0.114

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups.

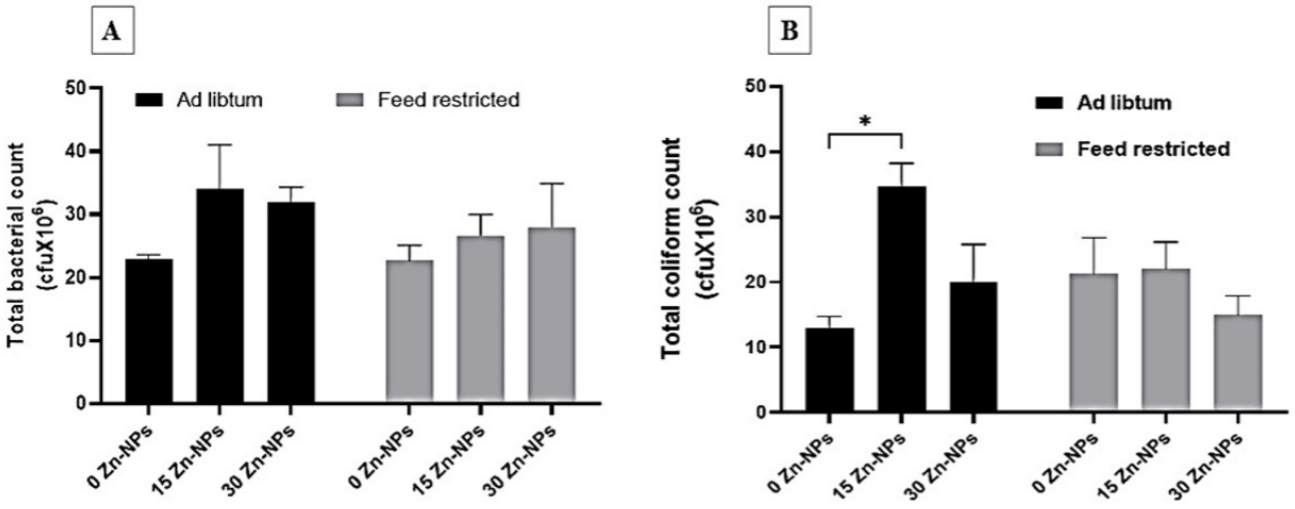

Figure 1: Effect of feed restriction and Zinc Nanoparticles supplementation on cecal microbiology (total bacterial count and total coliform count) in growing rabbits. Results expressed as means ± SEM

between them. The same response was obtained with the ALT enzyme activity. On the other hand, AST enzyme activity was significantly modified by the interaction of our treatments ($P < 0.05$). Zn supplementation lowered the AST concentration ($P < 0.05$) in feed restricted rabbits compared with their control rabbits without Zn addition. As illustrated in table 6, all the measured parameters of the serum lipid profile were significantly ($P < 0.05$) modified by ZnO-NPs addition in water. ZnO-NPs administration (both levels) to the full-fed rabbits resulted in lower ($P < 0.05$) TC, TG, LDL, VLDL, as well as reduced serum HDL only with 15 mg/L when compared to their control without Zn addition. Additionally, the same trend of reduction was observed in TC, TG, LDL, and VLDL in feed restricted rabbits which received the higher dose of ZnO-NPs (30 mg/L).

Volatile fatty acid concentration and caecal microbiology

In the freely fed rabbits, ZnO-NPs supplementation significantly reduced ($P < 0.05$) ammonia (AL-30) and individual VFAs concentrations (acetate, propionate, and butyrate) in the cecal contents at both levels (AL-15 and AL-30) compared to AL-0 (Table 7). The same response of

Table 8: Effect of feed restriction and Zinc Nanoparticles (ZnO-NPs) supplementation on morphology of small intestine (ileum) of growing rabbits

ZnO-NPs (mg/L)		Mucosal length (µm)	Villi length (µm)	Villi width (µm)	Crypt depth (µm)	Goblet cell (number/mm²)
AL-0		556.44±27.22^{bB}	290.57±19.27^{bB}	171.14±37.22^{aA}	72.04±10.62^{b}	2.78±0.25^{cD}
AL-15	*Ad libitum*	781.54±22.05^{aAB}	527.21±54.64^{aAB}	114.15±9.61b^{AB}	111.39± 4.46^{a}	8.71±0.04^{bB}
AL-30		826.11±12.33^{aAB}	239.07±115.45^{bB}	111.67±1.85b^{AB}	115.85±5.04^{a}	11.41±0.34^{aA}
R-0		673.19±32.19^{bB}	380.08±21.23^{bB}	85.30±4.80^{bB}	86.44±3.36^{b}	4.81±0.18^{cC}
R-15	Restricted	936.33±25.92^{aA}	709.36±35.20^{aA}	122.23±13.48^{bAB}	117.07±2.33^{a}	10.48±0.62^{bAB}
R-30		1000.93±83.95^{aA}	567.18±115.64^{abA}	126.37±10.03^{bAB}	104.89±2.44^{a}	11.48±0.32^{aA}
Two-way Anova (*P-value*)						
Feed offering regime		0.0021	0.015	0.162	0.565	0.0014
ZnO-NPs level		<0.0001	0.009	0.815	0.0002	<0.0001
Interaction		0.821	0.547	0.022	0.167	0.052

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups

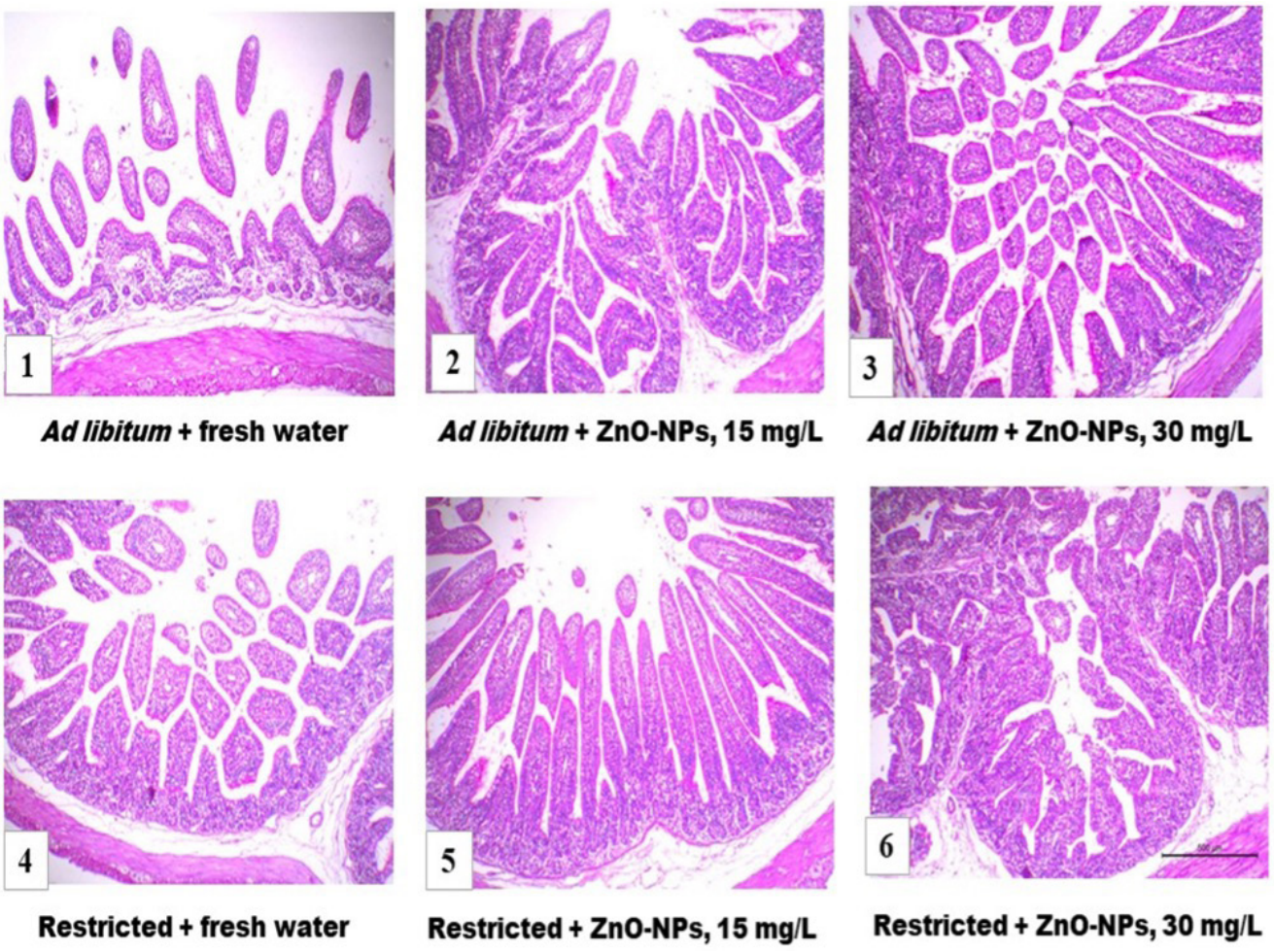

Figure 2: Morphology of rabbit ileum in response to different feeding regimes and ZnO-NPs supplementation As shown in 1) normal mucosal lining with no pathological changes; 2) an increase in the villi length; 3) an increase in mucosal length; 4) increase in the villi branches; 5) increase in the villi length; and 6) marked increase in the mucosal villi number and branches

reduction was obtained with cecal acetate and butyrate content in the feed restricted rabbits supplemented with 30 mg/L (R-30) when compared to control restricted rabbits (R-0).

The total bacterial count showed no differences among groups ($P > 0.05$) (Figure 1). On the other hand, the total coliform count was significantly altered ($P < 0.05$) with ZnO-NPs administration. In the freely fed rabbits, ZnO-NPs addition (15 mg/L) increased the total coliform count while reduced it ($P > 0.05$) when a higher dose of Zn was used (30 mg/L). In the restricted fed rabbits, non-significant difference was obtained ($P > 0.05$) when compared with the control receiving fresh water.

Intestinal Morphology

Intestinal morphometry parameters of the ileum are shown in Table 8 and Figure 2. Feed restriction and ZnO-NPs supplementation significantly affected the mucosal length, villi length, and goblet cell number. When compared to the control group, it increased ($P < 0.05$) mucosal length, villi length, crypt depth, and goblet cell number while decreasing villi width. Villi length was significantly increased in both rabbits (freely fed or restricted) and supplemented with 15 mg/L. On the other hand, villi length was reduced ($P < 0.05$) in the *ad libitum* fed rabbits supplemented with 30 mg/L of ZnO-NPs compared to rabbits supplemented with 15 mg/L ZnO-NPs. Goblet cells showed the highest number with the highest level of Zn added to the water. Besides, villi width was significantly changed in response to the interaction of the two factors ($P < 0.05$). In *ad libitum* rabbits, Zn administration reduced the villi width ($P < 0.05$), while showed no difference in the restricted rabbits ($P > 0.05$) compared with their control group without Zn addition.

Discussion

Feeding strategy for growing animals considers an important factor affecting their growth performance, lean body mass obtained and could help in avoiding problems associated with the early life fast growth rate such as increased body fat deposition, high incidence of metabolic disorders and high mortality. In recent years, global interest in using nanotechnology has been increased

as nanoparticles including Zn-NPs demonstrated a great potential as mineral supplements in livestock diets.

In the present study, feed restriction reduced rabbit growth performance in terms of final weight and gain while showed no effect on FCR. Different result was reported with Yakubu et al. (27), who found that feed restriction (8 hrs. per day feeding (7.00 a.m-3.00 p.m.) or a skip-a-day system) of weaned rabbits for five weeks resulted in no difference in their weight. Also, Meo et al. (5) found that both restricted (90 % of the *ad libitum*) and *ad libitum* rabbits showed similar weight at the slaughter time however, reported improved FCR in the restricted group. Birolo et al. (20) found that the application of 93% of *ad libitum* intake during the first growing period (from weaning, 37 d until slaughter (at 73 d and 80 d of age) enhanced rabbit health status without affecting growth performance. Moreover, feed restriction negatively affected the WG of rabbits, which could be associated with the reduced amount of feed consumed per day, which in turn resulted in inadequate intake of nutrients needed to support the rapid growth and development of the growing rabbit (28). No statistical difference was observed in the FCR between the different experimental groups. The reported results were consistent with Tumova et al. (29) who documented that feed restriction systems for growing rabbits had no effect on the feed efficiency. As reported before, the impact of feed restriction depends on its intensity, duration, digestive physiology, and age of rabbits when applied (30). Our results suggested that the restriction was not severe (mild restriction) to affect rabbit growth, resulting in restricted rabbit weight nearly approaching the weight of a full fed rabbit and reflected on the FCR at the end of the experiment.

Moreover, ZnO-NPs administration had no significant effect on rabbit growth performance, however, freely fed rabbits received 15 mg/L showed the highest body weight among groups. This improvement could be attributed to the better bioavailability and absorption of Zn, which has an important role in the metabolism of nutrients, in addition to the enhanced intestinal villi length. Unlike the obtained results, Hassan et al. (16) found that dietary supplementation of rabbits with nano-Zn at 30 and 60 mg /kg diet increased BW and FI. In support, Tag-El Din (15) reported that BW was insignificantly larger in rabbits that received different Nano-Zn compared with their control. Inconsistency in the obtained results between experiments could be associated with the difference in the experimental design, such as the route of Zn administration, nanoparticle size, number of samples used for measurements and the feeding regime applied in the current experiment.

Serum biochemical parameters are considered important diagnostic tools which help in assessing the metabolic condition of an animal and reflect its physiological response to different external and internal conditions, including nutrition (31). The interaction between the feed offering regime and Zn supplementation altered the albumin and globulin serum concentrations. The current results showed lower serum albumin concentration in the freely fed rabbits supplemented with ZnO-NPs (15 mg/L) compared to the same group subjected to feed restriction. In the same regard, Hassan et al. (16) found that diet fortification with nano Zn elevated total protein and globulin with no clear changes in albumin concentration of growing rabbits. Increased serum globulin in freely fed rabbits supplemented with ZnO-NPs was consistent with weaning piglets supplemented with nano Zn (32) and (33) in broiler chickens. Differences in the obtained results in terms of these parameters between restricted and freely fed rabbits could be associated with the reduced FI, which is correlated with the water consumption and consequently affected the dose of Zn administrated.

In the current experiment, a non-significant difference in glucose concentration in response to feeding restriction and ZnO-NPs addition was obtained. Likewise, Van Harten and Cardoso (34) found that the limitation of voluntary intake didn't reduce glucose concentration. They explained that animals under restriction require no more catabolism of glucose, and this was supported by the level of the glucose-6-phosphate, which didn't show any changes. Similarly, Ebeid et al. (35) stated that plasma glucose concentration showed no difference in growing rabbits subjected to feed restriction.

Regardless of the feed offering regime applied, serum MDA concentration was increased with increasing ZnO-NPs level added in water, suggesting that the addition of ZnO-NPs could have an oxidative stress effect on the growing rabbits. This finding was supported by Sharma et al. (36), who found that ZnO-NPs induced oxidative stress effects and increased the liver function enzymes, AST and ALT. In agreement, Ismail and El-Araby (37) found a significant increase in hepatic MDA levels in rabbits that received ZnO-NPs in their diet. Zn plays a significant role in the antioxidant defense system as an important part of the antioxidant enzyme SOD (38). The antioxidant defense system is formed of three levels of defense with SOD, CAT, and glutathione peroxidase forming the first level, as their main function is the prevention of free radical formation through scavenging their precursors (39). In our study, SOD and catalase serum activities were reduced by increasing the ZnO-NPs level, with the lowest concentration recorded at 30 mg/L, suggesting that the supplemented Zn failed to enhance the antioxidant system. Likewise, Ismail and El-Araby (37) found depressed renal and hepatic catalase activity in ZnO NPs supplemented rabbits, which could be associated with oxidative stress induced by nanoparticles. While this contradicts the findings of Hassan et al. (16) who reported elevated SOD activities in growing rabbits given 30 and 60 mg nano-Zn /kg diet.

Moreover, the interaction between the 2 factors affected the activity of the liver enzyme, AST. In *ad libitum* fed rabbits, the activity of this enzyme was increased with increasing Zn

levels, while the opposite result was obtained in restricted rabbits. Fazilati (40) reported that liver enzyme activity was increased in rat serum treated with ZnO nanoparticles (50 ppm, 100 ppm and 200 ppm). The measured kidney function related parameters, creatinine and uric acid, showed no changes between the different groups, suggesting no adverse effects of experimental treatments in the present trial. These results are consistent with Peris and Abd El-Latif (41), who found that ALT, urea-N, and creatinine serum concentrations showed no significant difference between restricted and *ad libitum* fed rabbits. Furthermore, ZnO-NPs supplementation at both levels was associated with a significantly increased serum Zn concentration in the Zn-supplemented rabbits, which suggests high availability and absorption of ZnO-NPs. Similar results were reported by Hassan et al. (16).

Regarding the lipid profile, it was altered with ZnO-NPs supplementation. In the full-fed rabbits, different lipid profile parameters, including TC, TG, HDL, LDL, and VLDL, were significantly reduced with Zn addition. The response of reduction in these parameters appeared with administration of ZnO-NPs (30mg/L) in the restricted rabbits. In the same direction, Tag-El Din (15) detected an insignificant reduction in plasma TG and TC of rabbits supplemented with Nano-Zn (30 and 60 mg/kg diet). Also, Ismail and El-Araby (37) who found a significant decrease in serum TG, TC, and VLDL-C in ZnO-NPs supplemented rabbits. Zinc consider as an important component of several enzymes (metalloenzymes) involved in lipid digestion and absorption (42). Additionally, cholesterol concentration responded to the interaction between the Zn added and the feed restriction regime as it showed the lowest level in restricted rabbits supplemented with 30 mg/L of ZnO-NPs. In support, El-Speiy et al. (30) documented lower TC, total lipids, and TG in rabbits under feed limitation. Our results suggested that ZnO-NPs added had an important regulatory role in the lipid metabolism, which was reflected by changes in the lipid profile parameters measured and growth performance of growing rabbits. The obtained lipid profile in response to ZnO-NPs administration needs further studies to understand the mechanism behind especially at the genetic level.

The fermentation process occurs in rabbit cecum and their end products as VFAs plays an essential role in feed utilization efficiency and rabbit performance. Gidenne (43) reported that the VFA covers about 30-50 % of the maintenance energy requirements of the adult rabbit. In the freely fed rabbits, ZnO-NPs addition in water altered the concentration of ammonia and different individual VFAs (acetic, butyric, and propionic) in the cecal content, as they were reduced compared with the control rabbits which received fresh water. A different result was obtained by Chrastinová et al. (44), who found no differences in VFAs profile between control and experimental groups supplemented with different sources of Zn. The obtained results confirm the importance of Zn as an essential microelement involved in metabolic processes in the body.

Rabbits subjected to a feed limitation regime showed a non-significant change in the total bacterial count or coliform count in the cecal content compared with the freely fed rabbits. Similarly, Martignon et al. (45) found that the FI level had no effect on the bacterial community structure (the number of bacterial 16S rDNA copies per gram of cecum). The constant composition of ingested feed material as well as the buffering capacity of the cecal contents were suggested by Michelland et al. (46) as the main explanation for the lack of effect of different FI levels on the cecal bacterial profile. In either full fed or restricted rabbits, ZnO-NPs administration showed a numerical increase in total bacterial count, with the higher count in the full fed ones. In support, Diao et al. (47) found a similar result when 100 mg/kg of zinc was supplemented in the diet of weaned piglets.

Besides, the total coliform count was increased with 15 mg/L ZnO-NPs administration, while it was reduced with 30 mg/L ZnO-NPs in the full-fed rabbits. In the restricted fed rabbits, non-significant difference was obtained when compared with the control receiving fresh water. In the same regard, Mahmoud et al. (18) found a significant reduction in the coliform count in the cecal content of broilers with different levels of Zn nanoparticles. The alteration in the cecal microbiota in response to the ZnO-NPs supplementation could be associated with its antibacterial activity (48). The latter author documented that the antibacterial activity of nanoparticles is dependent on their surface area and concentration, as the smaller the size, the larger the surface area, which becomes more reactive against bacteria, increasing their antibacterial activity. Therefore, the inconsistency obtained between the current study and the previous studies could be associated with the characteristics of nanoparticles used as size, concentration, form, and duration of exposure (18), species under the study, techniques that could give a complete picture of the microbial profile in the cecum and the host gut health and number of samples taken during the experiment. In the current study, the limited number of samples taken could contribute to the unclear picture of the experimental treatment response.

Nutrition including the amount of feed offered, is considered an important factor affecting the physiological function of the gastrointestinal tract as it relates to absorption (49). Gidenne et al. (2) assumed that the limitation of voluntary intake could change the morphology of the intestinal mucosa. In the present study, the ileal morphology of growing rabbits in terms of mucosal length, villi length, crypt depth, and goblet cell number were increased in response to feeding restriction and ZnO-NPs administration in water. In the same regard, Tůmová et al. (50) revealed that one-week feed restriction of growing rabbits modified the intestinal morphology as it showed longer small intestine villi with deeper crypts. In contrast, Martignon et al. (45) found that a three-week intake limitation applied (25% reduction of the

intake) did not affect the morphometric development of the ileal mucosa. The discrepancy in experimental outcomes might be associated with the restriction regime applied, duration, and intensity. Moreover, Oliveira et al. (51) stated that young animals have higher nutrient requirements and the intestinal mucosa recovers rapidly during refeeding. Dou et al. (52) reported that duodenal morphometric changes that occurred in response to starvation were normalized by refeeding on the second day. This could explain our finding of increased intestinal morphological parameters in restricted rabbits as they were freely fed in the first day then they were restricted in the next day (fed at the level of 93 % of *ad libitum* fed the day before), then again *ad libitum* followed by restriction.

Izadi et al. (53) reported that the increased intestinal absorptive capacity was associated with longer intestinal villi, and consequently beneficial effects on growth performance. This finding was shown in the present study as ZnO-NPs supplementation at the lower dose (15 mg/L) increased the intestinal villi length as well as deeper crypt, which was reflected by increased rabbits' final weight in the freely fed rabbits. In the same direction, El-Katcha et al. (33) reported that nano- Zn addition to the broiler diet increased the jejunal villi length. Increasing the Zn dose to 30 mg/L had a negative impact as it reduced the intestinal villi length in the freely fed rabbits or showed non-significant changes in the restricted rabbits when compared with the lower dose of ZnO-NPs (15 mg/L). These changes with the higher dose of Zn might be associated with reduced intestinal absorptive capacity and reduced body weight of rabbits obtained at the end of the experiment. Goblet cells contribute to the maintenance of the intestinal barrier by secreting mucin. In the present study, increasing ZnO-NPs levels were associated with an increased goblet cell number in the intestine, which could have a favorable protective effect on rabbit intestine. This result is in quite agreement with studies, which documented higher goblet cell numbers with Zn supplementation in mouse (54) and weaned piglets (55).

Conclusion

Under the conditions of the current experiment, it could be summarized that the feed restriction program applied in this experiment (rabbits were fed *ad libitum* for one day, then on the next day, they were fed at the level of 93 % of the intake fed the day before) altered rabbit growth performance (final body weight and weight gain with no differences in feed conversion ratio), improved the ileum morphology while had no effect on caecal fermentation (VFAs profile) or microbiological parameters. ZnO-NPs supplementation in both levels (15 and 30 mg/L water) differently modulated serum lipid profile, antioxidant enzymes and MDA, VFAs profile in cecum and ileal morphology with no differences in rabbit growth performance.

Disclosure statement

No conflicts of interest, financial, or otherwise, are declared by the authors.

Acknowledgements

Authors would gratefully thank Prof. Mosaad A. Soltan, Professor and head of Nutrition and Veterinary Clinical Nutrition Department, Faculty of Veterinary Medicine, Alexandria University, Egypt for his help during the course of the experiment.

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Vpliv omejitve krme in dodajanja nanodelcev cinkovega oksida na rastno zmogljivost, biokemijo krvi, črevesno morfologijo in parametre cekalne fermentacije rastočih kuncev

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Izvleček: V tej študiji smo proučevali odziv rastočih kuncev na omejitev krme in dodajanje nanodelcev cinkovega oksida (ZnO-NP) v okviru uspešnosti rasti, biokemičnih parametrov v serumu, morfologije črevesja in fermentacije v slepem črevesu. Skupno 60 samcev novozelandskih kuncev je bilo naključno razdeljenih v 6 skupin: AL-0 (krmljenje *ad libitum* + sladka voda kot kontrola); AL-15 (krmljenje *ad libitum* + voda z dodatkom 15 mg/l ZnO-NP) in AL-30 (krmljenje *ad libitum* + voda z dodatkom 30 mg/l ZnO-NP). Skupine R-0, R-15 in R-30 so bile enake prvim trem, vendar z omejenim režimom krmljenja. Kunci, hranjeni *ad libitum* z dodatkom ZnO-NP (15 mg/L), so imeli največjo telesno maso brez statistično značilnih razlik v primerjavi s skupinami AL-0, AL-30 in R-0. Razmerje pretvorbe krme (FCR) se med različnimi poskusnimi skupinami ni razlikovalo ($P > 0,05$). Dodajanje ZnO-NP je vplivalo na zmanjšanje parametrov lipidnega profila v serumu, tj. encim katalaza pri R-30 in superoksid dismutaza pri AL-15 in AL-30, medtem ko se je vsebnost serumskega malondialdehida (MDA) povečala tako pri kuncih, krmljenih *ad libitum*, in kuncih z omejenim režimom krmljenja. Dajanje ZnO-NP je pri AL-30 v primerjavi s kontrolo (AL-0) povzročilo znižanje vsebnosti amonijaka v slepem črevesu ter vsebnosti posameznih hlapnih maščobnih kislin (acetata, butirata in propionata) ($P < 0,05$). Kot odgovor na omejitev krme in dodajanje ZnO-NP so se spremenili morfološki parametri ileuma (dolžina sluznice, dolžina resic in število čašastih celic).

Ključne besede: omejitev krme; rast; kunci; nanodelci Zn oksida

DOI 10.26873/SVR-1611-2023
UDC 636.09:599.742.731:616.718.19-71
Pages: 149–54

Original Research Article

Retrospective Analysis of Extra-Pelvic Injuries Verified at the First Admission of Cats With Pelvic Fractures

Key words

extra-pelvic;
injury;
trauma;
skeleton

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Abstract: This retrospective study aimed to identify the common extra-pelvic injuries at the first admission at the hospital of cats with pelvic fractures. The medical records and radiographs were assessed. Seventy-three cats with pelvic fractures were identified, of which 41 were associated with extra-pelvic injuries. Of the 41 animals with extra-pelvic injuries, 21 were females and 20 were males. Motor vehicle trauma represented 56.09% of the pelvic fracture cause. Injuries to the appendicular skeleton included femur (n=12), tibia (n=1), lateral malleolus (n=1) and olecranon (n=1) fractures, and unilateral hip luxation (n=4). In the axial skeleton, mandibular condyle fracture (n=1), fracture of lumbar vertebrae (n=1), fracture and/or luxation of the coccygeal vertebrae (n=3), and luxation between S3 and the first coccygeal vertebra (n=3) were detected. Sacrum or sacroiliac fractures were detected in six cases. Sacroiliac luxation was verified in 22 cats unilaterally (n=15) and bilaterally (n=7). Respiratory tract lesions were pulmonary contusion (n=2), pneumothorax (n=2), and diaphragmatic hernia (n=1). Lesions of the urinary system organs included bladder rupture (n=3) and bladder entrapment in the hernia (n=1). In the integumentary system, there was one case of cutaneous laceration and one of subcutaneous emphysema. Traumatic hernias of the abdominal wall were found in five cats. Nervous system lesions included traumatic brain injury (n=2) and spinal cord injury (n=1). Two cats presented with constipation. Two animals died, and two were euthanized due to the severity of the injuries. In conclusion, the extra-pelvic injuries at admission were more frequent in the appendicular skeleton, mainly represented by femur fractures.

Received: 12 October 2022
Accepted: 17 April 2023

Introduction

Anatomically, the bony pelvis is constituted of the os coxae, which include the ilium, ischium, and pubis; the sacrum; and the first coccygeal vertebra (1, 2). The acetabulum, ilium, and sacroiliac joint are considered weight-bearing structures of the pelvis (1, 3). Including all trauma-induced fractures verified in the small animal practice it has been reported that pelvic fractures correspond to around 20-25% of those that occur in cats (2-5). The causes of pelvic fractures in cats include motor vehicle trauma or falls from heights, but there are reports of kicking and crushing injuries (3, 4).

Because the pelvic bones are protected by surrounding musculature, major trauma is needed to induce fractures in these bones, which also promotes extra-pelvic injuries (1). Consequently, various body systems may be affected in cats with pelvic fractures. These may include fractures of long bones, fracture/luxation of the sacroiliac joint, hip luxation, and herniation, among others (1, 4, 6, 7). For example, in a study of 108 fractures in cats detected over one year, 73% had hind limb involvement, and 22% included the pelvis/sacrum (8). In addition, a retrospective study of 280 cats with pelvic fractures observed involvement of the abdomen

(57.5%), thorax (49.6%), soft tissue (48.6%), nervous system (43.6%), extremities (25.4%), and face (13.9%) (9).

Since the evaluation should not be limited to the pelvis and immediate surrounding areas but the entire patient (4, 10), this retrospective study aimed to identify the principal extra-pelvic injuries verified at the first hospital admission of cats with pelvic fractures.

Materials and methods

This study was approved by the Institutional Ethics Committee for the Use of Animals (CEUA - no. 059/2020).

The medical records and radiographs of domestic cats with pelvic fractures examined at a veterinary teaching hospital were retrospectively assessed for a 6-year period. Data analysis included only those cats with extra-pelvic injuries concomitant to pelvic fractures verified at the first admission. Patient signalment (sex, breed, age, body mass), fracture cause, and types of extra-pelvic injuries (appendicular and/or axial skeleton and other body systems) were obtained. Radiographs of the pelvic fractures were classified (11) as follows: sacroiliac fracture/luxation, ilial wing fracture, fracture of the body of the ilium, acetabular fracture, ischial and/or tuber ischium fracture, and pelvic floor fracture. Unassisted death and euthanasia were also assessed.

Results

Seventy-three cats with pelvic fractures were identified, of which 41 were associated with extra-pelvic injuries (Table 1). Of the 41 animals with extra-pelvic injuries, 21 were females and 20 were males. Except for two Siamese cats, all the others were mixed breeds. Age ranged from 2.0 months to 15 years (mean of 25.72 months ± 40.49). Eight cats were classified as adults based on radiographs as age information was not available. The body mass ranged from 0.8 kg to 4.8 kg (mean of 2.8 kg ± 1.66), with one animal lacking this information.

Causes of pelvic fractures included motor vehicle trauma (56.09%; 23/41), dog bite (7.31%; 3/41), and domestic accidents (7.31%; 3/41), including trapping in a motor vehicle, falling from a height, and tile falling on the body. A total of 17.07% (7/41) of the cats lived indoors but escaped from home and returned injured. The history was not provided for 12.19% (5/41) of the cases. Twenty cases showed extra pelvic lesions exclusively in the skeletal system, 17 in other body systems, and four included the skeletal and other body systems.

Injuries to the appendicular skeleton included femur (n=12), tibia/lateral malleolus (n=2) and olecranon (n=1) fractures, and unilateral hip luxation (n=4). Femoral head was displaced into the pelvic canal because of the acetabular fracture in two cases (nos. 11 and 41). In the axial skeleton, mandibular condyle fracture (n=1), fracture of lumbar vertebrae (n=1), fracture and/or luxation of the coccygeal vertebrae (n=3), and luxation between S3 and the first coccygeal vertebra (Cd1) (n=3) were detected. Sacrum or sacroiliac fractures were observed in six cases. Sacroiliac luxation was verified in 22 cats unilaterally (right side, n=8; left side, n=7) and bilaterally (n=7). In addition, there were cats with traumatic brain injury (n=2) and spinal cord injury (n=1).

Respiratory tract lesions were pulmonary contusion (n=2), pneumothorax (n=2), and diaphragmatic hernia (n=1). Lesions of the urinary system organs included bladder rupture (n=3) and bladder entrapment in the hernia (n=1). In the integumentary system, there was one case of cutaneous laceration and one of subcutaneous emphysema. Traumatic hernias of the abdominal wall were found in five cats. Two cats presented constipation at admission.

Two animals died (nos. 26 and 41), and two were euthanized (nos. 21 and 38) due to the severity of the pelvic injuries, respectively associated with pulmonary contusion, constipation, L6 fracture, and brain injury/bladder rupture.

The individualized or combined site of the pelvic fracture in the 41 cats were as follow: ilial body (14.63%, n=6); ilial body and pelvic floor (7.31%, n=3); ilial body, pelvic floor and ischium/tuber ischium (14.63%, n=6); ilial body, pelvic floor and acetabulum (4.87%, n=2); ilial body and sacrum (2.43%, n=1); pelvic floor (4.87%, n=2); pelvic floor and sacrum (2.43%, n=1); pelvic floor and ischium/tuber ischium (24.39%, n=10); pelvic floor, ischium/tuber ischium and sacrum (4.87%, n=2); ischium/tuber ischium (7.31%, n=3); tuber ischium and sacrum (2.43%, n=1); acetabulum (4.87%, n=2); acetabulum and sacrum (2.43%, n=1); acetabulum and ischium (2.43%, n=1).

The pelvic floor fracture had an occurrence of 63.41% (26/41), followed by ischial/tuber ischium fracture (53.65%; 22/41), ilial body fracture (43.90%; 18/41), sacrum fracture (14.63%; 6/41), and acetabular fracture (14.63%; 6/41). Table 1 shows cats' signalment, fracture cause, extra-pelvic injuries, and site of the fracture/luxation.

Discussion

The present study evaluated extra-pelvic injuries at first admission in domestic cats with pelvic fractures and verified their occurrence in 56.16% (41/73) of cases. The proportion was lower than in a study of cats with pelvic fractures in which 93.6% (262/280) had an additional injury established by body regions, of which 82.1% were due to road traffic accidents and 10.4% high-rise syndrome (9). Although vehicular trauma was the most frequent cause in both studies, the high-rise syndrome characterized by

Table 1: Signalment (sex, breed, age, body mass) of 41 cats with extra-pelvic injuries resulting from pelvic fractures, including fracture cause and site of the fracture/luxation.

No.	Breed	Sex	Age	Body Mass	Cause	Extra-pelvic lesion	Fracture/luxation sites
1	mixed	F	6 mo.	1.8 kg	Motor vehicle trauma	Mid-diaphyseal, long oblique right femoral fracture	Bilateral ilial body fracture
2	mixed	F	6 mo.	1.4 kg	Trapping in a motor vehicle	Mid-diaphyseal, comminuted right femoral fracture (open)	Right ilial body fracture
3	mixed	M	2 yr.	4 kg	Motor vehicle trauma	Mid-diaphyseal, comminuted right femoral fracture	Bilateral pelvic floor fracture; Right sacrum fracture
4	mixed	F	1 yr.	4.7 kg	Motor vehicle trauma	Left distal femoral fracture (Salter Harris type II).	Right ischial fracture
5	mixed	F	4 mo.	2.2 kg	Motor vehicle trauma	Right distal femoral fracture (Salter Harris type I).	Left ilial body fracture, Left pelvic floor fracture, Left acetabular fracture; Right sacroiliac luxation S3–Cd1 luxation
6	mixed	M	4 mo.	2.4 kg	Motor vehicle trauma	Left proximal femoral physeal fracture	Bilateral pelvic floor fracture, Right ischial fracture; Bilateral sacroiliac luxation; Sacrum luxation (S2-S3)
7	Siamese	M	6 mo.	2.3 kg	Motor vehicle trauma	Right proximal femoral physeal fracture	Right acetabular fracture; Sacrum luxation (S1-S2, S2-S3)
8	mixed	M	6 mo.	2.5 kg	Motor vehicle trauma	Right proximal femoral physeal fracture	Right ilial body fracture S3–Cd1 luxation
9	mixed	M	1 yr.	3.8 kg	Escaped	Left proximal femoral physeal fracture	Right pelvic floor fracture, Right ischial fracture, Bilateral tuber ischium fracture
10	mixed	F	2.6 mo.	0.8 kg	Motor vehicle trauma	Right proximal femoral physeal fracture	Right pelvic floor fracture, Left sacroiliac luxation
11	mixed	M	11 mo.	2.3 kg	Escaped	Long oblique fracture of distal third of right tibia-fibula Right hip luxation	Left ilial body fracture, Bilateral pelvic floor fracture, Right acetabular fracture; Left sacroiliac luxation
12	mixed	M	1 yr.	3.8 kg	Escaped	Fracture of the left condylar process of the mandible Left ocular proptosis	Left ilial body fracture, Left pelvic floor fracture; Right sacroiliac luxation
13	mixed	F	6 mo.	2.3 kg	-	Left lateral malleolus fracture	Right ilial body fracture, Right pelvic floor fracture, Left ischial fracture
14	Siamese	F	Adult	3.7 kg	Motor vehicle trauma	Left hip luxation	Left pelvic floor fracture, Left ischial fracture
15	mixed	M	5 mo.	3.3 kg	Motor vehicle trauma	Right hip luxation	Left tuber ischium fracture; Bilateral sacroiliac luxation
16	mixed	F	4 yr.	4.8 kg	Motor vehicle trauma	Right hip luxation	Ilial body fracture; S3–Cd1 luxation
17	mixed	F	3 mo.	1.3 kg	Falling	L7-S1 subluxation, Cd6-Cd7 subluxation, Cd9 Fracture	Avulsion of the left tuber ischium; Bilateral sacroiliac luxation; Sacrum fracture (S2), S2-S3 subluxation
18	mixed	F	8 mo.	2.9 kg	-	Co1-Co2 subluxation	Right pelvic floor fracture, Right ischial fracture Left sacroiliac luxation
19	mixed	F	2 mo.	1.1 kg	Dog bite	Co6-Co7 luxation	Right acetabular fracture, Right ischial fracture Left sacroiliac luxation
20	mixed	F	1.5 yr.	3.7 kg	Motor vehicle trauma	L7-S1 articular process luxation	Left ilial body fracture
21	mixed	M	Adult	3.5 kg	Motor vehicle trauma	L6 vertebral body fracture	Bilateral pelvic floor fracture, Left ischial fracture; Bilateral sacroiliac luxation
22	mixed	F	6 mo.	2.5 kg	Motor vehicle trauma	Diaphragmatic hernia	Bilateral pelvic floor fracture, Avulsion of tuber ischium
23	mixed	M	3 yr.	3.8 kg	Escaped	Right hind limb proprioceptive deficit	Bilateral pelvic floor fracture; Right sacroiliac fracture/luxation, Left sacroiliac luxation

24	mixed	M	6 mo.	4.5 kg	Dog bite	Pulmonary contusion; Subcutaneous emphysema	Left acetabular fracture
25	mixed	M	Adult	5.2 kg	-	Bladder entrapment in the inguinal hernia	Bilateral pelvic floor fracture; Bilateral sacroiliac luxation
26	mixed	F	7 mo.	2.2 kg	Motor vehicle trauma	Pulmonary contusion Left proximal femoral physeal fracture	Bilateral pelvic floor fracture, Right ischial fracture; Right sacroiliac luxation
27	mixed	F	11 yr.	2.1 kg	Escaped	Pneumothorax Olecranon fracture	Left ilial body fracture, Left pelvic floor fracture, Left ischial fracture; Right sacroiliac luxation
28	mixed	M	Adult	4.4 kg	Motor vehicle trauma	Pneumothorax	Right ilial body fracture, Bilateral pelvic floor fracture, Right ischial fracture
29	mixed	F	10 yr.	4.5 kg	Motor vehicle trauma	Bladder rupture	Right ilial body fracture, Right pelvic floor fracture, Right ischial fracture
30	mixed	M	Adult	3.2 kg	Motor vehicle trauma	Traumatic brain injury	Left ilial body fracture, Right sacroiliac luxation
31	mixed	M	Adult	3.6 kg	Tile falling on the body	Spinal cord injury	Left tuber ischium fracture
32	mixed	M	2 mo.	1 kg	Motor vehicle trauma	Cutaneous laceration in the tail and right pelvic limb.	Left ilial wing and body fracture, Bilateral pelvic floor fracture, Bilateral tuber ischium fracture; Right sacroiliac luxation
33	mixed	F	4 yr.	3 kg	Motor vehicle trauma	Traumatic abdominal wall hernia	Bilateral pelvic floor fracture, Right ischial fracture; Bilateral sacroiliac luxation
34	mixed	F	7 mo.	2.6 kg	Motor vehicle trauma	Traumatic abdominal wall hernia	Left pelvic floor fracture, Avulsion of the right tuber ischium; Sacrum fracture (S2), S2-S3 subluxation
35	mixed	M	6 mo.	2 kg	-	Traumatic abdominal wall hernia Left proximal femoral physeal fracture	Left ilial body fracture, Bilateral pelvic floor fracture, Left tuber ischium fracture; Left sacroiliac luxation
36	mixed	F	Adult	4 kg	Escaped	Traumatic abdominal wall hernia	Right ilial body fracture; Sacrum fracture (S1-S2); Left sacroiliac luxation.
37	mixed	M	adult	3.6 kg	Motor vehicle trauma	Traumatic abdominal wall hernia	Left ilial body fracture, Bilateral pelvic floor fracture
38	mixed	F	15 yr.	-	Motor vehicle trauma	Traumatic brain injury Bladder rupture	Left ilial body fracture, Left pelvic floor fracture; Right sacroiliac luxation
39	mixed	M	2 yr.	3.3 kg	Escaped	Bladder rupture	Left pelvic floor fracture, Left ischial fracture; Right sacroiliac luxation
40	mixed	F	4 yr.	2.8 kg	-	Constipation	Left pelvic floor fracture, Left ischial fracture
41	mixed	M	3 mo.	2.1 kg	Dog bite	Pelvic narrowing, constipation	Left acetabular fracture; Sacrum fracture/ luxation (S1-S2); Bilateral sacroiliac luxation

falling from buildings was not verified in the current study, reflecting differences in environmental factors.

There were 36.58% (15/41) of cats with long bone fractures in this study, of which 29.26% (n=12) were in the femur. However, only two femoral fractures had a comminuted pattern. In a study with 103 cats, the type of pelvic fracture was not associated with the severity of femoral fracture (n=13), being most diaphyseal and comminute (6). The fact suggests that the mechanism of femoral injury was potentially more severe in those cases than in ours. On the other hand, this same study verified fractures involving the growth plate of the femoral head in cats with a mean age of 6.6 months (6). Similarly, cats with proximal femoral physeal fractures (n=7) in the present study had a mean age of 6.22 months, indicating a young population exposed to the same risk.

Pelvic fracture accompanied by sacroiliac luxation was noted in 53.65% of patients (22/41), 63.63% unilateral (n=14) and 36.36% (n=8) bilateral. The sacroiliac luxation should be treated surgically in bilateral injuries, with severe displacement or neurological deficits, among others (1). One study found that 65% (11/17) of cats had uni or bilateral sacroiliac subluxations concurrent with sacral fractures that were attributed to a possible weak attachment of the

pelvis to the vertebral column in the species (12). Sacrum or sacroiliac fractures were detected in 14.63% of the animals (6/41). The sacroiliac luxations or fractures may cause instability, pain, and neurologic lesion (5). An association between sacral body fracture and ischial body fracture was described in a study (6), but this association was not found in the present study.

Hip luxation was verified in 9.75% of the cats (4/41), two young cats and two adults, and only one related to acetabular fracture. In another study, both hip luxation and comminuted fracture of the femoral neck were found in cats with pelvic fractures and a mean age of 39.3 months (6). The presence of hip luxation makes the treatment method for pelvic fracture more complex (3).

Despite reports that sciatic nerve dysfunction can be detected in 11% of cats with pelvic fractures (1), only one cat showed a sensory deficit in one pelvic limb (2.43%) associated with sacroiliac fracture/luxation and bilateral pelvic floor fracture. Peripheral nerve injury was described in 11 cats related to sacroiliac fracture-dislocation, as verified in the present study, or may be due to the ilial fracture as described in the literature (13). In addition, an association between sciatic nerve injury and ipsilateral iliac body fracture has been reported (6).

Thoracic trauma can also occur in cats with pelvic fractures (1, 5) that promote injuries, such as pulmonary contusions, pneumothorax, hemothorax, rib fractures, and diaphragm rupture, among others (11). In the present study, 12.19% (5/41) of cats showed thoracic trauma, including diaphragmatic hernia, pneumothorax, and pulmonary contusion, most resulting from a car accident. The diagnosis and initial management of these injuries are fundamental due to the potential to become life-threatening (1, 14).

Concerning urinary tract injury, one incarcerated bladder and three bladder ruptures were observed. All cases of bladder ruptures were associated with concomitant pelvic floor fractures, which corresponded to 11.53% of all pelvic fractures. The bladder is one of the organs most damaged combined with pelvic fractures, which may be related to penetrating trauma from a fracture fragment or a full bladder being pressed or ruptured by blunt trauma (4, 7, 10). In addition to bladder rupture, ureteral avulsion, urethral laceration, and kidney injury have also been reported (1, 11), but these were not identified in the current study. The uroabdomen is classified as a medical emergency, and surgical repair of the lesion should be performed once the animal is stable (15).

Five (12.19%) traumatic hernias of the abdominal wall were detected in the present study. In addition to herniation, there are reports of rectum laceration and rupture of the pre-pubic tendon (3, 7) that were not observed in the current study. Obstipation and constipation are generally considered late complications, with a high risk in cases of severe pelvic narrowing (10, 11). Constipation was verified in two cats, probably related to the type of pelvic fracture and associated injuries, but also due to the time interval between the injury and admission to the hospital.

Euthanasia was done in two cats, one due to a spinal fracture and the other with traumatic brain injury and bladder rupture. A retrospective study of 208 cats with pelvic fractures observed a higher mortality rate in cases of neurological injuries or bilateral involvement of the weight-bearing axis (9). Acute death because of pelvic fractures is uncommon in cats, but mortality and morbidity associated with injuries can occur (10). In the current study, there were two deaths at presentation, one cat had a pulmonary contusion caused by a car accident, and the other had a sacral fracture and constipation due to a dog bite.

The highest number of fractures occurred in the pelvic floor (63.41%, n=26), followed by the ischial and/or tuber ischium fracture (53.65%, n=22) and the ilial body (43.90%, n=18). These findings differed from a study with 103 pelvic fractures, in which 90% of cats had fractures involving the pelvic floor, followed by ilial body fractures (48.5%) and ischial body fracture or avulsion of the tuber ischium (26%) (6). Since the ilial body is included in the weight-bearing structures of the pelvis, surgical management must be considered (1, 3).

Acetabular fractures accounted for 14.63% (n=6) of the lesions. The acetabulum is also a weight-bearing segment of the pelvis and must be reconstructed if fractures occur in its central and caudal areas (1, 3). In a study with 103 cats, 17.5% (n=18) had acetabular fractures. In general, the impact drives the femoral head into the acetabulum, with consequent acetabular fracture, which may be associated with ilium and pubis fractures (5). A severe lesion was observed in two cases (4.87%) because of the femoral head displacement into the pelvic canal.

The most frequent fracture combinations were pelvic floor and ischial/tuber ischium fracture (24.39%, n=10), followed by the ilial body, pelvic floor, and ischial fracture/avulsion of the tuber ischium (14.63%, n=6). The findings differed from another study in which the most frequent combination was the pelvic floor and ilial body fractures (6), suggesting different effects of trauma events on populations.

In conclusion, the extra-pelvic injuries at the first admission of cats with pelvic fractures were more frequent in the appendicular skeleton, mainly represented by femur fractures. Other lesions, such as in the respiratory or urinary systems, were in fewer numbers but represented potentially life-threatening conditions.

Acknowledgements

The authors would like to thank the National Council for Scientific and Technological Development (CNPq) for the PIBIC scholarship and Research Productivity Fellowship (PQ 301585/2017-2).

The authors declare that there are no conflicts of interest.

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Retrospektivna analiza poškodb izven medenice, potrjenih ob prvem sprejemu mačk z zlomom medenice

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Izvleček: Namen te retrospektivne študije je bil ugotoviti pogoste zunajmedenične poškodbe ob prvem sprejemu mačk z zlomom medenice v bolnišnico. Pregledali smo medicinsko dokumentacijo in rentgenske slike. Identificirali smo 73 mačk z zlomom medenice, od katerih jih je bilo 41 povezanih z zunajmedeničnimi s poškodbami. Od 41 živali z zunajmedeničnimi poškodbami je bilo 21 samic in 20 samcev. Poškodbe zaradi motornih vozil so predstavljale 56,09 % vzrokov zlomov medenice. Poškodbe privesnega skeleta so vključevale zlom stegnenice (n = 12), golenice (n = 1), lateralnega skočnega sklepa (n = 1) in olekranona (n = 1) ter enostranski izpah kolka (n = 4). V osnem skeletu so bili odkriti zlom čeljustnega kondila (n = 1), zlom ledvenih vretenc (n = 1), zlom in/ali izpah repnih vretenc (n = 3) ter izpah med S3 in prvim repnim vretencem (n = 3). V šestih primerih so bili ugotovljeni zlomi križnice ali križnice in črevnice. Izpah križnično-črevničnega sklepa je bil potrjen pri 22 mačkah, enostransko (n = 15) ali obojestransko (n = 7). Poškodbe dihalnih poti so vključevale kontuzijo pljuč (n = 2), pnevmotoraks (n = 2) in diafragmalno hernijo (n = 1). Poškodbe organov sečil so vključevale rupturo mehurja (n = 3) in ujetje mehurja v hernijo (n = 1). Najden je bil en primer raztrganine kože in en primer podkožnega emfizema. Travmatske hernije trebušne stene so bile ugotovljene pri petih mačkah. Poškodbe živčnega sistema so vključevale travmatsko poškodbo možganov (n = 2) in poškodbo hrbtenjače (n = 1). Dve mački sta imeli zaprtje. Dve živali sta poginili, dve sta bili zaradi resnosti poškodb evtanazirani. Zaključimo lahko, da so bile zunajmedenične poškodbe ob sprejemu pogostejše na privesnem skeletu, ki so jih predstavljali predvsem zlomi stegnenice.

Ključne besede: zunajmedenični; poškodba; travma; skelet

DOI 10.26873/SVR-1686-2023
UDC 636.09:614.253.4:591.4:612.75:159.953.5
Pages: 155–60

Original Research Article

An Insight Into Veterinary Students' Perceptions on the use of 3D-Printed Bone Biomodels in Anatomy Learning

Key words

veterinary anatomy education;
3D printing;
bone biomodel;
student perspective

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Abstract: Today, conventional teaching methods are losing their effectiveness at transferring knowledge and skills, prompting the presentation of alternative strategies that hold more promise. One of the innovative alternative education materials in veterinary anatomy education is the models produced on three-dimensional (3D) printers. The subject of this study is 4 different bone biomodels 3D modeled and printed with reference to cadaver-derived bones. In the study, a total of 298 students were asked to evaluate these biomodels in terms of their similarity to the reference bones. According to the survey, 75.5% of the students stated that their biomodel resembled the reference bones. In addition, 64.8% of these students stated that the use of biomodels can be efficient in learning the skeletal system. These outcomes showed that a sample from each of the 4 main bone types could be replicated on a 3D printer with an acceptable similarity ratio. Based on student opinions about these four different biomodels, we think that 3d printed biomodels deserve to be evaluated as an alternative in anatomy education.

Received: 18 January 2023
Accepted: 5 July 2023

Introduction

It is commonly admitted that anatomy education is a very significant field in both human and veterinary medicine (1). The main purpose of anatomy education is to provide students with required basic information of the field via effective learning and teaching methods (2). In theoretical and practical anatomy classes, methods such as verbal expression, visual expression (e.g., slide shows, training videos), software containing 3D visuals, virtual reality, and augmented reality, are widely utilized (3-5). Besides the mentioned methods, educational materials such as cadavers are utilized in practical anatomy education to increase the quality of the training. Plaster, clay, or plastic models, and plastinates, which are acquired from cadavers, are also of use (6-8).

While the computer simulations or atlas pictures, which are used in anatomy training, appeal only to the visual attention of the students, plastinates, clay or plastic models, and cadavers attract visual attention and provide a tactile experience. Educational materials, which are procured from cadavers, provide students with the opportunity to know actual anatomical variations as well as get visual and tactile information (9). Besides the ethical concerns, supplying the educational materials from dead organisms is difficult and the amount of the material is generally inadequate. Furthermore, smell, texture, and students' awareness about the fact that the materials are procured from dead organisms, cause a significant degree of motivational decrease in the learning process/success of the students. One of the innovative alternative educational materials in applied anatomy education is the models produced on three-dimensional printers. Three-dimensional printers, which are regarded as future technology, offer various advantages in terms of material production by going beyond conventional manufacturing methods. 3D printing is defined as the physical output of a 3D design. It is considered different from the conventional production methods which are based on cutting, punching, and molding. These models

are claimed to become realistic alternatives of educational materials in comparison with the both conventional models and cadavers (10-12).

The use of 3D printers and adaptation of the 3D outputs in anatomy training is an up-to-date and original subject that is still being studied (13-15). In our study, 4 different biomodels were produced based on cadaver-derived bones. The biomodels were presented to the students for review along with the bones. Students were asked to compare the similarity of biomodels with cadaver bones. In this study, it was aimed to investigate the potential of biomodels as an alternative educational tool by using the student's point of view.

Materials and methods

Reference materials of the study were obtained from the bone archive of Sivas Cumhuriyet University, Faculty of Veterinary Medicine, Anatomy Department. All stages of biomodel production of reference bones were carried out in the Biomodel Laboratory of Sivas Cumhuriyet University, Faculty of Veterinary Medicine, Andrology Department. The method of the study includes the stages of preparing reference bones, 3D modeling and processing of data sets, printing the biomodel on a 3D printer, and statistical evaluation of the models.

Preparing samples

For the production of biomodels, equidea humerus was selected as the sample of long bones; equidae ossa coxae was selected as the sample of flat bones; an equidea 3rd phalanx was selected as the sample of short bones; and finally, an equidea 3rd cervical vertebra was selected as sample of irregular bones. Using these cadaver-derived reference bones, 3D bone biomodels were produced through replication.

3D modeling and processing data sets

Optical scanning method was utilized to obtain 3D volumetric data of each selected bone. Upon placing the reference bones on the scanning bench, datasets of 0.5mm precision were obtained from the bones which were 360° scanned continuously via a 3D scanner (Structure Sensor, Occipital Inc, USA). The object file format (.obj) data sets were transferred to the computer. The initial analysis of the data sets was carried out via modeling software (3D Builder, Microsoft Corporation) and unnecessary details were eliminated. Upon completing the initial analysis, the evaluation of the data sets was carried out via the computerized 3D graphics software (ZBrush, Pixologic). Thanks to this software, geometrical deviations, which stem from the 3D scanning process, were controlled. Biomodels were visually compared with reference bones in terms of their similarities/differences. The redundant data points were

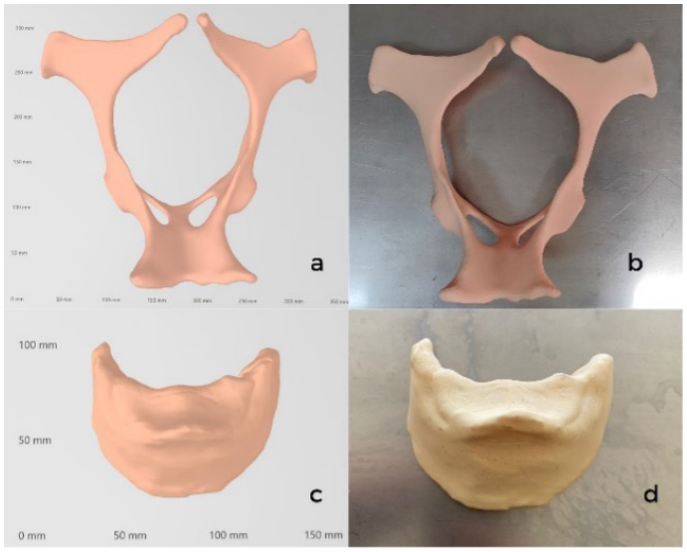

Figure 1: Appearance of specimens with flat and short bones. a; coxae digital model b; coxae biomodel c; 3rd phalanx digital model d; 3rd phalanx biomodel

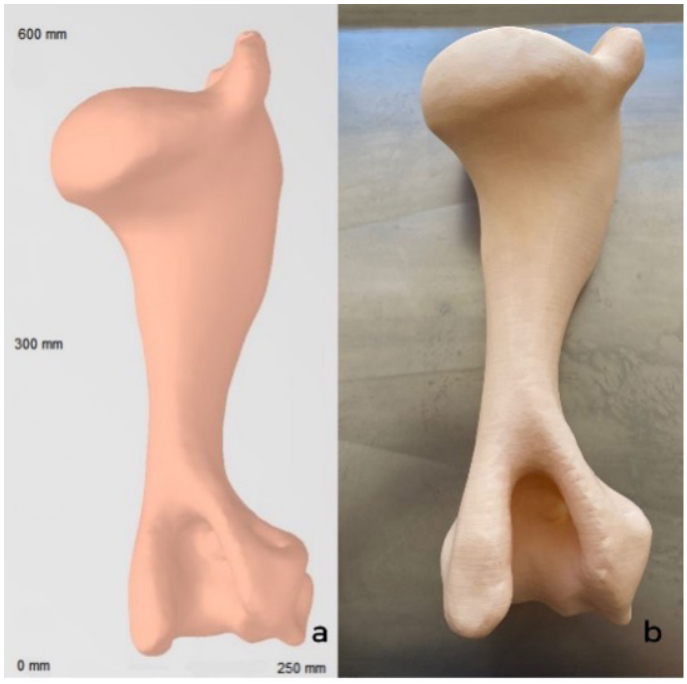

Figure 2: Appearance of specimens with long bones. a; humerus digital model b; humerus biomodel

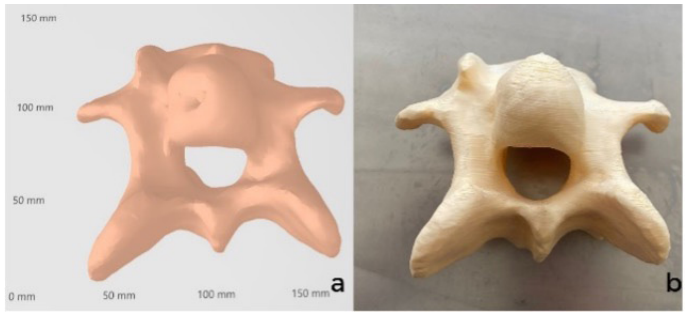

Figure 3: Appearance of specimens with irregular bones. a; 3rd cervical vertebra digital model b; 3rd cervical vertebra biomodel

removed and a single continuous mesh was created. 3D models and reference bones were compared by the anatomist. It was checked whether the 3D printed models had important anatomical features as in the bone from which they were referenced. For the similarity control of the biomodels, 8 criteria for humerus, 7 for coxae, 5 for phalanx and 5 for vertebra were considered. Incomplete-insufficient parts on the 3D models have been edited (e.g. smoothing of facies articularis in the phalanx model). To convert the complete data sets to 3D printing compatible format (gcode). The data sets were transferred to the rendering software (ideaMaker 4.0.1, Raise 3D Technologies, Inc.) of the 3D printer.

3D printing

Rendered data sets were transferred to the 3D printer (Raise 3D N2 Plus, Raise, USA). For the 3D printing process, previously tested and approved poly-lactic acid (PLA) filament (nGen, Colorfabb) was used (Figure 1,2,3). The printing parameters were selected as layer height of 0.28 mm and printing speed of 40 mm/s.

Gathering data

The data of the study was gathered via a quantitative descriptive method. For this purpose, a total of 298 students, who took the Anatomy course of Sivas Cumhuriyet University, Faculty of Veterinary Medicine, were included in the study without sampling. In the faculty where the study was carried out, anatomy course is taught as a total of 12 hours, 4 hours of which is theoretical lectures and 8 hours are practical lectures. While textbooks and atlases are used in the theoretical part of the course, cadavers and plastic models are used in the practical part. The reference bones and biomodels were given to the participants and the participants were asked to examine both groups of materials. Following this step, a Likert scale survey, which measures the attitude of the students towards applied anatomy class and used materials, was filled by the participants (Appendix 1).

Evaluation of the data and statistical analysis

The data obtained from the students were analyzed using SPSS Statistics v.23 for Windows. The students were asked to choose the most suitable one among these options. For the positive items, the options were scored on a Likert-type scale (5 = strongly agree, 4 = agree, 3 = undecided, 2 = disagree, 1 = strongly disagree). When interpreting the arithmetic means, it was considered that the mean values between 1.00 and 1.80 were of value to the extent of strongly disagree, that those between 1.81 and 2.60 were of value to the extent of disagreeing, that those between 2.61 and 3.40 were of value to the extent of undecided, that those between 3.41 and 4.20 were of value to the extent of agree, and that those between 4.21 and 5.00 were of value to the extent of strongly agree. Proportional data were analyzed by using the chi-square test. The method for collecting and evaluating data was designed as double-blind.

Research ethics committee approval

For the study, required approvals were obtained from Sivas Cumhuriyet University Non-Invasive Clinical Research Ethics committee (Res. Number: 2018/10-20)

Results

In the study, a survey was applied to a total of 298 participants, 108 of whom were female and 190 of whom were male. In this survey, the participants' opinions about practical anatomy class, used class materials, and use of biomodels were gathered. When the students' responses for the statements in the attitude scale were evaluated, it became clear that only 21.2% of the participants found the practical anatomy classes adequate (Table 1).

On the other hand, 49.7% of the participants stated that they are disturbed by the smell when they use bones in practical classes (Table 2). In the attitude scale, when the feedback given by the participants to the statements about the use of bio-models is examined, the rate of the participants who find biomodels similar to bones appears to be 75.5% (Table 3).

In addition to these, the rate of the participants who find the use of biomodels beneficial in learning the skeletal system is 64.8% (Table 4).

According to the feedback given for the statement, for which the participants were asked to rate the materials in the applied anatomy classes according to their efficiency levels, it was observed that the most efficient material after the bones is the biomodel (Figure 4).

Table 1: Distribution of the feedback for the statement "Practical classes are adequate"

	n	%
Strongly disagree	42	14.1
Disagree	75	25.2
Partly agree	118	39.6
Agree	53	17.8
Strongly agree	10	3.4
Total	298	100

Mean: 2.71 , Std. Deviation: 1.02.

Table 2: Distribution of Participants' Feedback for the Statements about Bone Use (%)

Statements	Strongly Disagree	Disagree	Partly Agree	Agree	Strongly Agree	Mean	Std. Deviation
Disturbed by the smell of bone.	11.1	18.1	21.1	26.2	23.5	3.32	1.31
Disturbed by the texture of bone.	24.2	33.2	25.2	9.1	8.4	2.44	1.19
Disturbed by bones' belonging to a dead animal.	45.6	30.2	12.8	6.0	5.4	1.95	1.14

Table 3: Distribution of Participants' Feedback for the Statements about Biomodel Use (%)

Statements	Strongly Disagree	Disagree	Partly Agree	Agree	Strongly Agree	Mean	Std. Deviation
Can distinguish the species on biomodels.	3.4	7.0	23.2	45.3	20.8	3.73	0.97
Biomodels resemble the bones.	3.7	2.3	18.1	46.6	28.9	3.94	0.94

Table 4: Distribution of Participants' Feedback to the Statement "Utilizing biomodels in learning skeletal system is efficient"

	n	%
Strongly disagree	21	7,0
Disagree	20	6.7
Partly agree	63	21.1
Agree	89	29.9
Strongly agree	104	34.9
Total	298	100

Mean: 3.79 , Std. Deviation: 1.19.

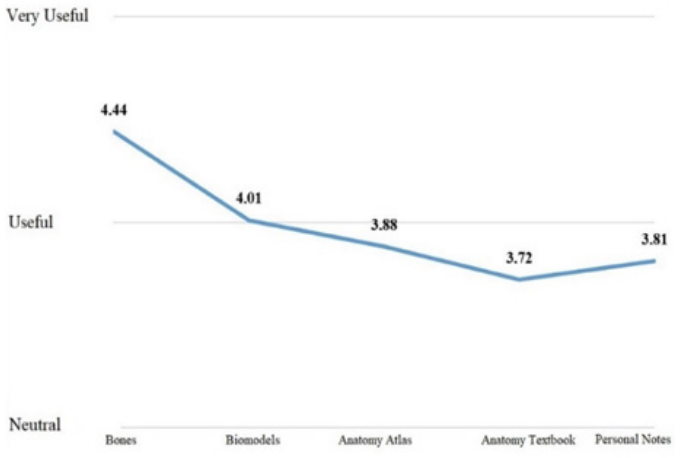

Figure 4: Distribution of the average of utility rating of educational materials according to student attitudes (1: Useless – 5: Very Useful)

Discussion

The success level of veterinarians in their professional life depends on learning skills in many practical fields during their education/training. Anatomy class is the basis of the long training process leading to veterinary medicine (16). In veterinary faculties, students are first faced with the anatomy course. Knowledge of anatomy is acquired with considerable difficulty. In anatomy education, in addition to the quality of the theoretical courses in veterinary faculties, the education standards of practical classes are also tried to be increased and enriched to overcome the educational difficulties (17). The preparation of cadaver poses a variety of challenges, including ethical dilemmas, moral concerns, and complications known as the dissection experience. The development of students clinical skills is directly related to the diversity of the mentioned educational materials. However, obtaining these materials is significantly challenging in financial, ethical, legal, and cultural terms (18, 19). Furthermore, these bones must be processed with a series of chemical solutions to be able to make the bones available for the use of students (20). Formaldehyde and other chemicals, which are the components of these solutions, pose a significant level of threat to the health of both students and trainers (21). Due to the possible hazards of the chemicals which are used in the preparation of practice materials, the use of cadavers is restricted by legal regulations (22). It was determined that only 21.2% of the students find the applied anatomy lessons sufficient in this study when the opinions of the participants to the statements were evaluated on the attitude scale. However, encountering cadavers and bones in the anatomy class causes concerns with the students. Furthermore, this situation leads some students to avoid or to skip practical classes (23, 24). This outcome is not confusing, because the reason for preferring veterinary faculty is undoubtedly the love of animals and the desire to help them for many students.

While anatomy education requires as many samples and varieties of bones as possible, it is also under pressure of perspectives and regulations which prioritize animal welfare. Many anatomy trainers still believe that the ideal form of anatomy education can be provided only through classic cadaver-bone samples which are prepared via methods that have been practiced for longer than a century. However, the search for an alternative method to animal use for educational purposes has become a rising perspective (25, 26). Alternative educational tools such as dummies and models have begun to be utilized in basic classes like Morphology and Andrology (4, 27). Until recently, the inadequate details of these models were a major problem for this field. Biomodels present a novel option for advancing the field of veterinary anatomy education. In this study, the ratio of participants, who perceive the biomodels successful in terms of resembling the reference bones, appears to be 75.5%. In addition, the ability to recognize animal species from bone samples has been demonstrated with high success (79.5%) by the students participating in this study. This showed us that the replication of the reference bones was achieved at a sufficient level. The high-level resemblance of biomodels to reference bones can contribute to the motivation and learning success of students. On the other hand, considering that 49.7% of the participants are disturbed by the smell while using bones in practical classes, the advantage of utilizing biomodel stands out even further. Kucukaslan et. al. reported that the students are particularly hesitant in touching pig, cat, and dog cadavers (28). The fact that the biomodels are similar to the ones and not taken from live animals increases the motivation of students to learn by touching. In another study stated the idea that animal use in biomedical studies is essential (29). This idea is supported by the results of another study, which claims that the most effective learning material is bone. In that study, 28.4% of the participants claimed that personal notes such as sketches are the best learning tools for anatomy class, and 23.7% of them stated that they found the use of textbooks useful, while 14.4% of the participants regarded the anatomy atlas as the best option. Moreover, the ratio of the participants who found plastic models efficient was only 5.4%. In this study, biomodels were interestingly found to be more effective than textbooks and anatomy atlas, and biomodels were stated as the second most useful tool after bone. This outcome stems from the utilization of innovative materials and technologies. Participants adopting such an attitude supports the literature perspective which states that the transfer of knowledge through biomodels will help in clinic practice (1, 17).

Thanks to the 3D printers; production costs will be reduced, and it will be possible to provide each student with an individual biomodel. In addition to the mentioned advantages of biomodels, it must also be remembered that biomodels can also be utilized in other classes such as Pathology, Surgery, Radiology, and Reproduction. However, the utilization of biomodels in anatomy training also has some limitations and disadvantages. The experience gained from dissection practice and necropsy training cannot be substituted adequately by these biomodels (30). The creation of biomodel collections for a variety of animal species within veterinary science will require a significant amount of time. The inability to model small structures (bones of the middle ear) and to maintain color harmony are the main limitations. However, studies on 3D modeling and printing of these structures continue (31). It is predicted that developments will be achieved in this field parallel to the developments in imaging and image processing technologies.

Conclusion

As a result, in our current environment, in which science and technology are developing rapidly, the transfer of knowledge and skills via traditional teaching methods is also losing its validity and plausible alternative strategies are put forward. The use of biomodels should be considered as an alternative that can increase the efficiency of the training process in veterinary medicine. Furthermore, the use of biomodels in practical classes can be of help in relieving the anxiety and discomfort of students.

In this study, it has been shown that biomodel replicas from 4 different cadaver bones can be produced with an innovative method, each with its own anatomical criteria. Moreover, it was concluded that the students deemed these biomodels remarkably alike to their references and approved of their implementation. We think that diversifying education with alternative learning tools will benefit all stakeholders of veterinary medicine, especially students.

Acknowledgements

The authors declared that there is no conflict of interest.

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Vpogled v mnenje študentov veterine o uporabi 3D-tiskanih bioloških modelov kosti pri učenju anatomije

A. Koçyiğit, H. H. Ari, B. A. Uslu

Izvleček: Danes običajne metode poučevanja izgubljajo svojo učinkovitost pri prenosu znanja in spretnosti, zato bi bilo potrebno spodbujati alternativne, bolj obetavne strategije. Eno od inovativnih alternativnih učnih gradiv pri pouku veterinarske anatomije so modeli, izdelani na tridimenzionalnih (3D) tiskalnikih. Predmet te študije so štirje različni biološki modeli kosti, pripravljeni s 3D modeliranjem in tiskanjem, osnovani na kosteh, pridobljenih iz trupel. V študiji je bilo skupaj 298 študentov naprošenih, da ocenijo te biološke modele glede na njihovo podobnost s pravimi kostmi. 75,5 % študentov je navedlo, da je njihov biološki model podoben referenčnim kostem. Poleg tega je 64,8 % teh študentov izjavilo, da je uporaba bioloških modelov lahko učinkovita pri učenju skeletnega sistema. Ti rezultati so pokazali, da je mogoče vsakega od 4 glavnih tipov kosti kopirati na 3D-tiskalniku s sprejemljivim razmerjem podobnosti. Na podlagi mnenj študentov o teh štirih različnih bioloških modelih menimo, da bi 3D-tiskani biološki modeli lahko bili vrednoteni kot alternativni pripomoček pri izobraževalnem procesu anatomije.

Ključne besede: poučevanje anatomije v veterini; 3D tiskanje; biološki model kosti; perspektiva študentov

DOI 10.26873/SVR-1788-2023
UDC 639.111.2:591.16:577.21(234.323.6)(497.4)
Pages: 161–72

Original Research Article

First Insight Into Genetic Diversity of Alpine ibex (*Capra ibex*) in Slovenia

Key words

Capra ibex;
mitochondrial DNA;
MHC *DRB* exon 2;
reintroduction;
management

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Abstract: In Europe, the Alpine ibex (*Capra ibex*) was on the brink of extinction in the 19th century. Therefore, different conservation measures were implemented, and several reintroductions were made in the Alpine arc, starting from the only surviving population in Gran Paradiso, Italy. An extreme historical bottleneck and additional reintroductions have strongly shaped the genetic make-up of recent populations, resulting in significant genetic drift and profound inbreeding across the species range. To support science-based conservation actions, molecular methods have been increasingly used. However, such analyses did not include populations in Slovenia.We analysed neutral loci (partial fragment of mitochondrial cytochrome b, mtDNA) and the adaptive major histocompatibility complex (MHC *DRB* exon 2) of the Alpine ibex from both Slovenian populations (Julian and Kamnik-Savinja Alps) to understand how past reintroductions and recent management have affected the genetic diversity of the species. Results showed that both populations are genetically severely depleted, carrying only one mtDNA haplotype and one functional allele for MHC *DRB* exon 2, Caib-DRB*01. This calls for further conservation actions, including the reintroduction of individuals with different genetic background. However, the Alpine ibex is currently considered a non-native species in Slovenia, which makes conservation actions extremely difficult and threatens the long-term survival of the species. Therefore, scientists and population managers are urging policy/decision makers to change the status of the species to the native one and consequently to allow reintroductions. These appeals are supported by previous archaeological data on the existence of bones assigned to Alpine ibex in the Julian Alps, and evidence of severe genetic depletion in current ibex populations confirmed in this study.

Received: 9 June 2023
Accepted: 14 August 2023

Introduction

Due to centuries-long intensive hunting, the Alpine ibex (*Capra ibex*) was on the brink of extinction in the 19th century (1). However, in the 20th century several reintroduction programmes were conducted in which captive-bred individuals from the only surviving population in Gran Paradiso, northern Italy, were translocated to various locations across the Alps (2, 3). As a result of these efforts, the Alpine ibex

has successfully recovered, and the number of individuals has increased from <100 to >53,000 within a century (4). The stepwise reintroduction strategies have been multidirectional and have included both primary translocation from captive breeding and secondary translocations from previously established populations (3, 5, 6, 7). Subsequent rounds of reintroductions and rapid increase in abundance have strongly shaped the genetic make-up of populations, and their isolation has also contributed to further genetic differentiation causing gradual genetic substructure in established (sub)populations (8, 9, 10).

Previous genomic studies revealed that the surviving population from Gran Paradiso has maintained a higher level of genetic variability compared to reintroduced populations, primarily due to the series of bottlenecks experienced by the reintroduced populations during translocations (9, 10). Moreover, new populations established by only a small number of individuals showed an increase in genetic drift and inbreeding, which leads to additional loss of genetic variation, reduces the efficacy of natural selection, and increases the expression of deleterious recessive mutations (8, 11). By analysing the genomic footprint and the consequences of sequential bottlenecks, Grossen et al. (10) found evidence for the concurrent purging of highly deleterious mutations and the accumulation of mildly deleterious ones. This suggests that recolonization bottlenecks induced both relaxed selection and purging, thus reshaping the landscape of deleterious mutation load. The accumulation of deleterious mutations was significantly lower in populations of >1,000 individuals in comparison with smaller populations (10). Maintaining an adequate effective population size of the Alpine ibex is hence of paramount importance for the species conservation (12, 13).

Current populations of Alpine ibex exhibit extremely low heterozygosity (8, 9, 10) and variability in major histocompatibility complex (MHC) genes (14). Introgression of the domestic goat *DRB* 2 allele (Caib-DRB*2) has been confirmed both in reintroduced populations in the Swiss Alps and in the source population of Gran Paradiso (15). In genetically depleted populations, introgression of the goat *DRB* allele likely reflects adaptation, as introgression increased the MHC *DRB* diversity. Based on genetic methods, Giacometti et al. (16) confirmed that wild Alpine ibex interbred with the domestic goats (*Capra hircus*) in a population in the southern Swiss Alps. The online survey recently performed by Moroni et al. (17) also showed that hybrids are present in most of the Alpine countries and that their occurrence is not a sporadic event, with some groups comprising 4–20 probable hybrids. The offspring of the hybrids are generally larger and heavier, have longer horns, some males do not have the characteristic horn folds, and their coat hair is darker (17). As one of the conservation actions in the Swiss Alps, all wild goats, including their hybrid offspring, were removed between 1998 and 2001 to protect the genetic purity of the Alpine ibex (16, 17).

According to archaeological records, the Alpine ibex occurred in the area of present-day Slovenia during the last glaciation, when it inhabited most of Europe, including the lowland areas of France, Luxembourg, the Czech Republic, Slovakia, Romania, Hungary, and Slovenia (18). After the last glaciation with the succession of vegetation in the lowlands, the range of the species was restricted exclusively to the Alpine arc (4, 18). Although some bone remains indicated the presence of the Alpine ibex in Slovenia in the Late Pleistocene (19, 20) and Holocene (21, 22), the species is currently recognised as non-native in the country. This definition or perception is based on the fact that there is no reliable data on the historical occurrence of the Alpine ibex in the Slovene Alps (23, 24). In contrast to conservation measures/projects implemented in other countries of the Alpine arc (summarised in (14)), the recognised non-nativity of the species in Slovenia makes its conservation (almost) impossible and therefore poses a threat to its long-term existence. However, a recent analysis of ancient DNA confirmed that four bone remains from the Julian Alps dated to the $5^{th}/6^{th}$ century were indeed a part of the Alpine ibex skeleton, which scientifically support the appeal for reconsidering the formal status of the species, i.e., to classify and manage it as a native species (25).

The Alpine ibex is the least common wild ungulate in Slovenia, with a population size estimated at about 300 individuals (26, 27). The species has certainly been present in Slovenia since 1890, when Baron Born established a colony of 20 ibex in the Karavanke Mountains, northern Slovenia. During both World Wars, the colony experienced two severe declines; in spite of three reintroduction events in the 1950s and 1970s, this colony disappeared in the early 1990s (23). Currently, Alpine ibex is present in two Slovene areas: the Kamnik–Savinja Alps and in Julian Alps. In the Kamnik–Savinja Alps, 4 ibex from Switzerland were released in 1953, followed by an additional 8 (4 males, 4 females) from Switzerland (park Sankt Gallen) in 1961 and 1965, and 7 from Gran Paradiso in 1967. This population reached its peak with >80 individuals in 1991, but after two outbreaks of sarcoptic mange (in 1991 and 2011) the population size declined to 30–35 individuals in 2022 (28). In the Julian Alps, 24 individuals from Gran Paradiso were released in the Triglav National Park between 1963 (1964) and 1966; population reached its peak of 330 individuals in 1996, followed by a rapid decrease due to sarcoptic mange outbreaks, with the minimum around 100 individuals in 2003 (26, 29), and a population size of 150–160 individuals in 2022 (30, 31). In the most western part of the Slovenian Julian Alps, i.e., outside the Triglav National Park, eight ibex (two from Gran Paradiso and six from Switzerland) were also released in the 1970s (31), and since 2000, immigration of individuals from the Italian side of the Kanin mountain has been confirmed (32). In 2022, the population size across the Julian Alps was assessed at 250 individuals, with 50 of them being present on the Slovene side of the Kanin mountain (31).

The genetics of Alpine ibex in Europe and its connection with ecology and spatial distribution are relatively well-known thanks to several large-scale studies (8, 9, 10, 33). Numerous sets of microsatellite loci have been developed for the species, both for studying genetic diversity and levels of inbreeding (8, 34, 35, 36, 37), as well as the close link MHC complex (14, 15, 37). The microsatellite markers were also proved to be useful for confirming hybridization events with domestic goats (16). In the last decade, modern genomic analyses have also been performed on the Alpine ibex, including single nucleotide arrays and whole genome sequencing (10, 38, 39). Unfortunately, however, none of these analyses included Alpine ibex from Slovenia.

In our study, we used neutral loci (partial fragment of mitochondrial cytochrome b, mtDNA cytb) and the adaptive major histocompatibility complex (MHC *DRB* II exon 2) to analyse the genetic diversity of two Alpine ibex populations in Slovenia, i.e., from the Kamnik–Savinja Alps and the Triglav National Park (Julian Alps). Specifically, we explored whether past management is reflected in the genetic architecture and how the different reintroduction strategies influenced the genetic diversity of populations. We hypothesized that the two populations would show depleted genetic diversity compared to the source population from Gran Paradiso due to the founder effect and inappropriate conservation actions, i.e., as the Alpine ibex has the status of a non-native species, which has prevented additional reintroductions aimed at ensuring connectivity between populations as well as adding new individuals to existing populations.

Materials and methods

Study area and sampling

To assess the genetic diversity of the two Alpine ibex populations from Slovenia, we analysed DNA from 33 samples: 10 from the Kamnik–Savinja Alps and 23 from the Julian Alps, respectively. DNA was extracted either from bones collected from skulls or muscle tissue taken from carcasses found between 2002 and 2020 (Fig. 1, Table S1). Samples from both areas were collected by professional gamekeepers from the Triglav National Park and the Slovenia Forest Service. In addition, to compare the genetic diversity of the Slovenians with other wild and captive populations, we

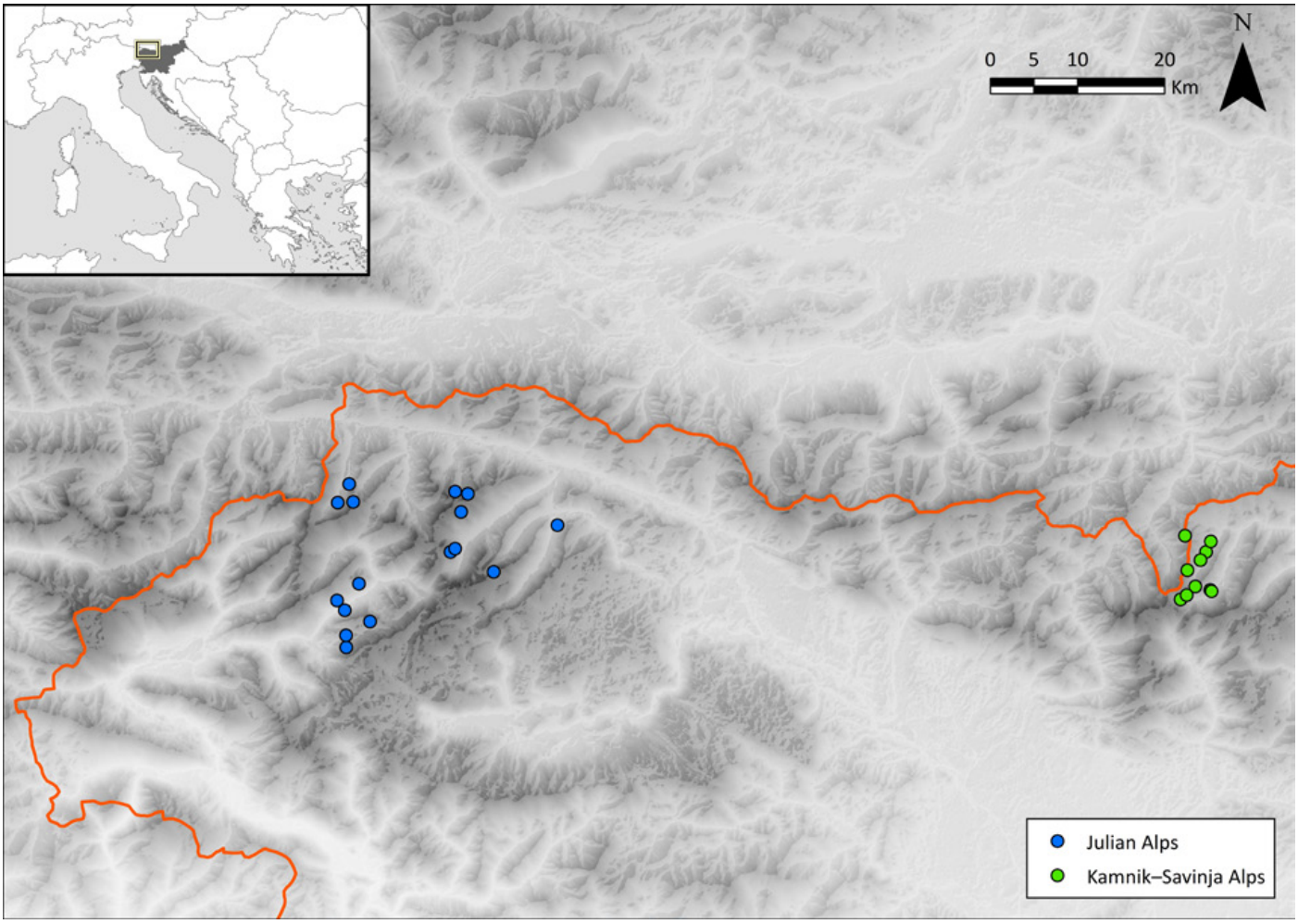

Figure 1: Sampling locations of Alpine ibex in Slovenia, period 2002–2020 (blue dots: Julian Alps (Triglav National Park); green dots: Kamnik–Savinja Alps); see Table S1 for details on the studied individuals and names of the localities

included in the analysis 17 blood samples of Alpine ibex from the Ljubljana Zoo (Slovenia; colony was established by male and female from Wildpark Feldkirch, Austria, and male and 3 females from Switzerland, unknown location), 5 tissue samples from Hohe Tauern (Austria), and 24 tissue or blood samples from Gran Paradiso (Italy).

DNA extraction and quality control

Bone samples from skulls were extracted with the High Pure Viral Nucleic Acid Kit (Roche, Switzerland), using the modified extraction method for highly degraded samples, developed and described by Rohland et al. (40). We used the E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek, USA) to isolate DNA from blood and tissue samples. The quality of extracts was assessed with the Qubit 3.0 using Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, USA).

Amplification and statistical analysis of the mitochondrial cytochrome b region (cytb)

The partial mitochondrial cytochrome b gene (cytb; 623 bp) was amplified using the universal primer set L14724: 5'-CGAAGCTTGATATGAAAAACCATCGTTG-3' and H15347: 5'-GATGGGTTATTTGATCCTGTTTCGTG-3' (41, 42, 43). All polymerase chain reactions (PCR) were performed in a 20 µl reaction mix, using Platinum Direct PCR Universal Master Mix (ThermoFisher Scientific, USA) and amplified on a Thermal Cycler 2720 (Applied Biosystems, USA). After denaturation 3 min at 95°C, 35 PCR cycles with 30 s at 95°C, 45 s at 61°C and 60 s at 72°C were performed, followed by a final extension step of 10 min at 72°C. Sanger nucleotide sequencing was performed on the SeqStudio Genetic Analyzer (ThermoFisher Scientific, USA) using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems, USA).

The CodonCode Aligner 4.27 (CodonCode Corporation, USA) was used to align the forward and reverse sequences. The resulting consensus sequences were aligned in MEGA 11 (44). The regions analysed in this study were combined with three previously published data downloaded from GenBank. Genetic diversity was estimated with haplotype diversity (Hd) and nucleotide diversity (π). All parameters were assessed with the programme DnaSP v.6.12 (45). The relationship among haplotypes was evaluated by constructing a median-joining haplotype network (46), using the PopART (47).

We included into the median-joining haplotype network three published nucleotide sequences of the Alpine ibex cytb from GenBank (nb. EU368877, AF034735, FJ207526), 23 sequences of samples from the Julian Alps and 10 from the Kamnik–Savinja Alps (Slovenia), 5 from Hohe Tauern (Austria), 24 from Gran Paradiso (Italy), and 17 from captive animals in Ljubljana Zoo (Table S1).

Amplification and statistical analysis of the major histocompatibility complex (MHC)

The Platinum Direct PCR Universal Master Mix (ThermoFisher Scientific, USA) was used to amplify the second exon of the MHC class II DRB gene using primers HL030: '5 -ATCCTCTCTCTGCAGCACATTTCC-3' and HL032: '5 -TCGCCGCTGCACAGTGAAACTCTC-3' (48). We performed PCR amplification in triplicate in 20-µl reaction mixtures (for details, see (49)).

The amplicons from the triplicates were pooled and purified with magnetic particles Agencourt® AmPure® (Agencourt Bioscience Corporation, A Beckman Coulter Company, USA), following the manufacturer's instructions. Concentrations of pooled and purified amplicons were quantified by Qubit 3.0 fluorometry using Qubit dsDNA BR (Broad range) Assay Kit reagents (ThermoFisher Scientific, USA). Samples were normalized to 3 ng and combined into a final library, which was again purified with Agencourt® AmPure® magnetic particles. For the separation, sizing and quantification of dsDNA final library of amplicons we used Agilent DNA High Sensitivity Kit on a 2100 Bioanalyzer (Agilent, Santa Clara, USA), according to the manufacturer's recommendations. We normalised the library to 100 pM, which was then multiplicated and bound with Ion Sphere particles (ISPs) using the Ion 520 & 530 Kit-OT2 reagent kit (ThermoFischer Scientific, USA) according to the protocol for sequencing 400 bp long fragments on Ion Torrent One Touch 2 (OT2) and sequenced following the ThermoFisher Scientific platform instructions on Ion Torrent S5, using the Ion 530 chip (ThermoFisher Scientific, USA).

For allele calling, we used the pipeline of the Amplicon Sequence Assignment (AmpliSAS) tool developed for high-throughput genotyping of duplicated polymorphic genes, such as MHC (50). The script was installed locally to analyse all the reads. Filtering of the raw data was performed with AmpliCLEAN by removing reads with a Phred quality score <20 and filtering all reads <250 bp and >300 bp. AmpliSAS clusters true variants with their potential artefacts based on platform-specific error rates. We used AmpliSAS's default parameters for Ion Torrent sequencing technology: a substitution error rate of 0.5 % and an indel error rate of 1 %. An accurate length was required to identify the dominant sequence within a cluster. We did not expect more than two *DRB* variants per individual, so we kept the "minimum dominant frequency" clustering threshold at 25 %, based on previously published works on Alpine ibex (14, 15, 37, 51). We discarded variants with a frequency <1 % within an amplicon. True variants of the *DRB* exon 2 fragments were aligned and translated into protein sequences to check for evidence of pseudogenes, such as the presence of premature stop codons or indels. A maximum of 200,000 reads per amplicon were used for allele calling. Since the web version of the AmpliSAS tool utilises only the first 5000 sample reads, the genotyping process was repeated with the same

parameters using the AmpliSAS script installed locally to analyse all reads.

The unique sequences were aligned, edited, and confirmed to be Alpine ibex MHC *DRB* exon 2 alleles by comparing them with alleles downloaded from GenBank (Table S1; GenBank nb. AY70631 from Albris, Switzerland) using MEGA 11 (44).

Results and discussion

Mitochondrial genetic diversity

We successfully sequenced mtDNA cytb from 78 out of the 79 samples (i.e., all except one from the Julian Alps). In the samples from Gran Paradiso, we detected haplotype H1 (already deposited in GenBank; Table S1) in 18 samples; three samples had haplotype H2, two samples had haplotype H3, and one had haplotype H4. The new haplotypes H2, H3 and H4 were deposited in GenBank under accession numbers OQ745823–OQ745825. Populations from the Julian Alps and the Kamnik–Savinja Alps had only the H1 haplotype. Haplotype diversity for all analysed samples was Hd = 0.431. The Alpine ibex populations revealed extremely low nucleotide diversity (π = 0.0008), possibly attributed to the historical bottleneck.

The median-joining network of mtDNA cytb haplotypes shows a star-shaped topology. The most common haplotype (H1) belongs to all studied populations. Alpine ibex from the Julian Alps and the Kamnik–Savinja Alps belong to the central, most common haplotype H1. Alpine ibex from Pointe de Calabre, France (sequences obtained from GenBank), Gran Paradiso (Italy), Hohe Tauern (Austria), and Ljubljana Zoo also share the same mitochondrial sequence. Haplotypes H2, H3 and H4, which belong only to the Gran Paradiso population, differ from the central haplotype only by one substitution (Fig. 2).

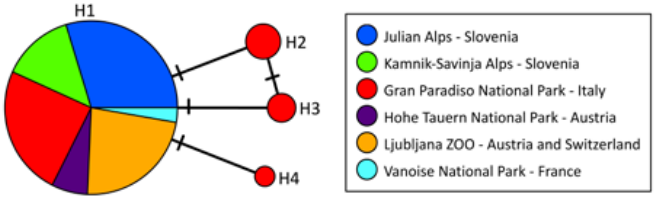

Figure 2: Median-joining network of mtDNA cytb haplotypes of the analysed Alpine ibex individuals. The size of the circles is proportional to the frequency of the haplotype, while the colours identify the area of the sample origin. The number of mutations separating the nodes is represented by lines crossing the branches of the grid.

MHC genetic diversity

We successfully analysed MHC *DRB* exon 2 from all 34 samples analysed, i.e., 24 from Gran Paradiso and 10 from the Julian Alps, Slovenia. We found one functional allele for MHC *DRB* exon 2, previously described by Schaschl et al. (48). No evidence of multiple locus amplification was found, confirming previous reports for Alpine ibex (52, 53). The samples from Gran Paradiso and the analysed Slovene population (Triglav National Park) had the same functional allele for MHC *DRB* exon 2, Caib-DRB*01, and we did not observe the presence of the allele Caib-DRB*02 in the studied populations. This allele was found by Grossen et al. (15) in a genetically severely depleted population of the Alpine ibex in Switzerland. The authors concluded that the introgression of the Caib-DRB*02 allele from domestic goats into the Alpine ibex was most likely due to adaptation, as introgression increased the diversity of the *DRB* gene in the MHC complex. The Caib-DRB*02 allele is otherwise identical to the *DRB* allele of the domestic goat (the so-called 'goat-like' *DRB* allele).

Possible consequences of low genetic diversity of Alpine ibex in Slovenia

Both mitochondrial and MHC genetic variability of Alpine ibex in the two Slovenian populations (the Julian Alps and the Kamnik–Savinja Alps) are very low. We found only one (the same) mtDNA cyt b haplotype and one MHC *DRB* exon 2 allele in both populations. Our results confirmed the low genetic variability of the Alpine ibex populations previously reported in France, Switzerland, and Italy (8, 14, 35). The presence of only one mtDNA haplotype and one MHC allele is likely the result of the founder effect but also of sequential bottlenecks in the 20th century due to improper management and the lack of connectivity among populations (23). Biebach and Keller (54) found that in Alpine ibex, the initial bottleneck reduced allele numbers more than subsequent bottlenecks, as predicted by theory (55, 56, 57). The preferential loss of low-frequency alleles is consistent, and a substantial proportion of alleles must be lost. If only higher frequency alleles remain in a population after an introduction, fewer founder individuals are required in subsequent reintroductions to retain most of the alleles present in the initial population. Thus, additional bottlenecks can reduce genetic variation even in the absence of an additional loss of alleles.

(Re)introductions and management history are the main determinants of today's genetic structure of the Alpine ibex in Slovenia; more than a hundred years after the first (re) introduction programmes we recorded depleted genetic diversity, which could lead to a severe population decline in the future (25). For example, in populations with low genetic variability, there is a risk of low disease resistance. In this respect, it is important to note that both in the Triglav National Park (Julian Alps) and in the Kamnik–Savinja Alps, periodic population declines were observed in the past due to increased mortality from infection with the scabies mite (*Sarcoptes scabiei*) (26, 28, 29, 31). Moreover, in ibex, sarcoptic mange has negative effects on the reproductive performance of both males and females, as already reported in Iberian ibex (*Capra pyrenaica*) (58, 59, 60).

The management of the Alpine ibex in Slovenia is in complete contrast to other successful managements throughout the Alpine arc. So far, >170 introduction events have been carried out in the Alps, leading to a dramatic increase in the abundance and spatial distribution of the species (4). Alpine ibex numbers in Europe have increased from only about 100 surviving individuals in the 19th century to >53,000 individuals, with an estimated population size increase of >400 % between 1975 and 2016, and the spatial distribution increase of 342 % between 1960 and 2020 (summarised in (61)). In contrast to this, the currently recognised non-nativity of the species in Slovenia hampers conservation efforts as reintroductions and releases of new individuals are not allowed throughout the ibex habitat as it completely overlaps with the Natura 2000 Network. This poses a severe threat to the long-term conservation of the species in Slovenia (25), particularly because genetic diversity (both mitochondrial and in immunogenes) is very low as revealed by our study. Therefore, there is an urgent need to change the status of the species, and subsequently implement active conservation/management, including new reintroductions, the success of which should be constantly monitored by the use of genomics tools to study the footprint and changes/improvement of Alpine ibex genetic diversity after a new conservation/management approach.

Conclusion

The study on mitochondrial genetic diversity of the Alpine ibex populations in Slovenia revealed limited haplotype variation, with only one predominant haplotype (H1) present both in the Julian Alps and the Kamnik–Savinja Alps populations. The analysis of MHC genetic diversity in the same populations showed very limited variability, with only one functional allele (Caib-DRB*01) present. Our findings highlight the negative effects of management history on the genetic structure of the Alpine ibex in Slovenia. The depletion of genetic diversity would probably lead to additional population declines and reduced disease resistance. The non-nativity status of the species in Slovenia hampers conservation efforts, preventing reintroductions and new releases throughout the ibex habitat. To ensure the preservation of Alpine ibex in Slovenia, urgent action is required to change the species' status and implement active conservation and management strategies, including reintroductions. Genomic tools should be utilized to monitor the genetic diversity of the population and evaluate the success of conservation efforts over time. Such measures are essential to safeguard the future of the Alpine ibex in the region.

Acknowledgements

Samples from Gran Paradiso (Italy) were collected in the framework of the long-term monitoring program going on in Gran Paradiso National Park. We thank Bruno Bassano and the park rangers for collecting and providing the samples. We are also thankful to the gamekeepers of the Triglav National Park and the Slovenia Forest Service for collecting samples.

Originality statement: The material submitted for publication has not been published except in abstract form, and it is not currently under consideration for publication elsewhere.

Ethical statement: All bone samples taken from the trophies used in the study were legally harvested during regular hunting activities prescribed by the state of Slovenia in annual wildlife management plans. No animals were sacrificed for the purpose of obtaining samples for this study.

Competing interest: The is no competing interest.

Author's contributions: Conceptualization: E.B., B.P.; sample providing: A.B., S.H., P.B., I.M.; sequences providing: N.P, S.H., I.M.; laboratory and statistical analyses: L.D., A.B., E.B.; writing – draft preparation: EB.; writing – review and editing: E.B., B.P., A.B., L.D., S.H., N.P., P.B. I.M.; funding: E.B. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by: (i) the Triglav National Park; (ii) the Slovenian Research Agency (programme group P1-0386); (iii) the STEPCHANGE European Union's Horizon 2020 Research and Innovation Program under grant agreement No. 101006386, oriented to accelerate collaboration with hunters as citizen scientists in wildlife/biodiversity monitoring and research.

Data availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Table S1: Data on Alpine ibex samples included in the study

Lab/GenBank ID	Sample ID	Country	Location	Year of sampling	Lat	Long	Cyt b hpt	MHC allele
LME2748	ALPKOZ- 3	Slovenia	Julian Alps – Kriški podi – pod Kolenom	2019	46.402	13.801	H1	/
LME2749	ALPKOZ- 4	Slovenia	Julian Alps – Jalovec	2017	46.421	13.680	H1	Caib-DRB*01
LME2750	ALPKOZ- 5	Slovenia	Julian Alps – Kriški podi – Gorenja luknja	2014	46.405	13.808	H1	/
LME2751	ALPKOZ- 6	Slovenia	Julian Alps – Kriški podi – pod Šplevto	2001	46.402	13.801	H1	/
LME2752	ALPKOZ- 7	Slovenia	Julian Alps – Kriški podi – pod Kolenom	2019	46.402	13.801	H1	Caib-DRB*01
LME2753	ALPKOZ- 8	Slovenia	Julian Alps – Jalovec	2008	46.421	13.680	–	Caib-DRB*01
LME2754	ALPKOZ- 9	Slovenia	Julian Alps – Jalovec	2011	46.421	13.680	H1	Caib-DRB*01
LME2756	ALPKOZ- 11	Slovenia	Julian Alps – Plazi – pod Pejči	2015	46.315	13.698	H1	/
LME2757	ALPKOZ- 12	Slovenia	Julian Alps – Kriški podi – Stružnik	2018	46.402	13.801	H1	/
LME2758	ALPKOZ- 13	Slovenia	Julian Alps – Plazi – pri Skali	2012	46.343	13.696	H1	Caib-DRB*01
LME2759	ALPKOZ- 14	Slovenia	Julian Alps – Kriški podi	2020	46.343	13.696	H1	Caib-DRB*01
LME2760	ALPKOZ- 15	Slovenia	Julian Alps – Kriški podi – pod Debelo pečjo	2009	46.392	13.933	H1	Caib-DRB*01
LME2761	ALPKOZ- 16	Slovenia	Julian Alps – Plazi – na Laberju	2009	46.343	13.696	H1	/
LME2762	ALPKOZ- 17	Slovenia	Julian Alps – Plazi – pri Skali	2019	46.343	13.696	H1	/
LME2763	ALPKOZ- 18	Slovenia	Julian Alps – Plazi – pod Risjem	2019	46.315	13.698	H1	Caib-DRB*01
LME2764	ALPKOZ- 19	Slovenia	Julian Alps – Plazi – Pejča	2019	46.318	13.683	H1	Caib-DRB*01
LME2765	ALPKOZ- 20	Slovenia	Julian Alps – Kriški podi	2002	46.380	13.834	H1	/
LME2766	ALPKOZ- 21	Slovenia	Julian Alps – Kriški podi – Korito	2004	46.429	13.710	H1	Caib-DRB*01
*	TNPCapr aIbexSI	Slovenia	Julian Alps – Krn	/	46.265	13.660	H1	/
*	WILD1C apralbex SI	Slovenia	Julian Alps – Krn	/	46.265	13.660	H1	/
*	WILD2C apralbex SI	Slovenia	Julian Alps – Krn	/	46.265	13.660	H1	/
*	WILD3C apralbex SI	Slovenia	Julian Alps – Bavšica	/	46.369	13.628	H1	/
*	WILD4C apralbex SI	Slovenia	Julian Alps – Log pod Mangartom	/	46.411	13.594	H1	/
LME3986	Kozorog 1	Slovenia	Kamnik–Savinja Alps	2013	46.366	14.562	H1	/
LME3987	Kozorog 2	Slovenia	Kamnik–Savinja Alps	2008	46.366	14.562	H1	/
LME3988	Kozorog 3	Slovenia	Kamnik–Savinja Alps	2013	46.366	14.562	H1	/
LME3989	Kozorog 4	Slovenia	Kamnik–Savinja Alps	2011	46.366	14.562	H1	/

Table S1: Continued

Lab/GenBank ID	Sample ID	Country	Location	Year of sampling	Lat	Long	Cyt b hpt	MHC allele
LME3991	Kozorog 6	Slovenia	Kamnik–Savinja Alps	2008	46.366	14.562	H1	/
LME3992	Kozorog 7	Slovenia	Kamnik–Savinja Alps	2008	46.366	14.562	H1	/
LME3993	Kozorog 8	Slovenia	Kamnik–Savinja Alps	2019	46.366	14.562	H1	/
LME3996	Kozorog 11	Slovenia	Kamnik–Savinja Alps	2011	46.366	14.562	H1	/
LME3997	Kozorog 12	Slovenia	Kamnik–Savinja Alps	2010	46.366	14.562	H1	/
LME3995	Kozorog 10	Slovenia	Kamnik–Savinja Alps	2013	46.366	14.562	H1	/
LME3660	O01Q	Italy	Orco	2017	45.402	7.510	H2	Caib-DRB*01
LME3661	O01R	Italy	Orco	2018	45.402	7.510	H1	Caib-DRB*01
LME3662	O02Q	Italy	Orco	2017	45.402	7.510	H1	Caib-DRB*01
LME3663	O02R	Italy	Orco	2018	45.402	7.510	H1	Caib-DRB*01
LME3664	O03Q	Italy	Orco	2017	45.402	7.510	H3	Caib-DRB*01
LME3665	O03R	Italy	Orco	2018	45.402	7.510	H1	Caib-DRB*01
LME3666	V01N	Italy	Valsavarenche	/	45.582	7.218	H1	Caib-DRB*01
LME3667	V01P	Italy	Valsavarenche	2016	45.582	7.218	H1	Caib-DRB*01
LME3668	V02N	Italy	Valsavarenche	/	45.582	7.218	H1	Caib-DRB*01
LME3669	VO2P	Italy	Valsavarenche	2016	45.582	7.218	H3	Caib-DRB*01
LME3670	VO3P	Italy	Valsavarenche	2016	45.582	7.218	H2	Caib-DRB*01
LME3671	V04N	Italy	Valsavarenche	/	45.582	7.218	H1	Caib-DRB*01
LME3672	V04P	Italy	Valsavarenche	2016	45.582	7.218	H1	Caib-DRB*01
LME3673	V04Q	Italy	Valsavarenche	2017	45.582	7.218	H1	Caib-DRB*01
LME3674	V05N	Italy	Valsavarenche	/	45.582	7.218	H1	Caib-DRB*01
LME3675	VO5Q	Italy	Valsavarenche	2017	45.582	7.218	H4	Caib-DRB*01
LME3676	V06N	Italy	Valsavarenche	/	45.582	7.218	H2	Caib-DRB*01
LME3677	V06Q	Italy	Valsavarenche	2017	45.582	7.218	H1	Caib-DRB*01
LME3678	V07N	Italy	Valsavarenche	2017	45.582	7.218	H1	Caib-DRB*01
LME3679	V08N	Italy	Valsavarenche	/	45.582	7.218	H1	Caib-DRB*01

Table S1: Continued

Lab/GenBank ID	Sample ID	Country	Location	Year of sampling	Lat	Long	Cyt b hpt	MHC allele
LME3681	V08Q	Italy	Valsavarenche	2017	45.582	7.218	H1	Caib-DRB*01
LME3682	V09Q	Italy	Valsavarenche	2017	45.582	7.218	H1	Caib-DRB*01
LME3683	V11N	Italy	Valsavarenche	/	45.582	7.218	H1	Caib-DRB*01
LME3684	V20L	Italy	Valsavarenche	2018	45.582	7.218	H1	Caib-DRB*01
EU368877(62)	CiGP1	Italy	/	/	/	/	H1	/
*	AIB4574 CapraIb exAT	Austria	Döllach Hohe Tauern	2013	46.980	12.939	H1	/
*	AIB4832 CapraIb exAT	Austria	Hohe Tauern	2016	47.163	12.505	H1	/
*	AIB5332 CapraIb exAT	Austria	Heiligenblut Hohe Tauern	2017	47.014	12.808	H1	/
*	AIB5333 CapraIb exAT	Austria	Heiligenblut Hohe Tauern	2017	47.014	12.808	H1	/
*	AIB5336 CapraIb exAT	Austria	Heiligenblut Hohe Tauern	2017	47.014	12.808	H1	/
*	ZOO17C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO18C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO19C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO20C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO28C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO29C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO30C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO31C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO40C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO42C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO43C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO47C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/

Table S1: Continued

Lab/GenBank ID	Sample ID	Country	Location	Year of sampling	Lat	Long	Cyt b hpt	MHC allele
*	ZOO48C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO49C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO52C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO56C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
*	ZOO58C apralbex SI	Austria and Switzerland	ZOO Ljubljana	/	46.054	14.472	H1	/
AY70631(48)	/	Switzerland	Albris, Kanton Graubuenden	2005	46.658	9.608	/	Caib-DRB*01
FJ207526(63)	Cyto 2002-037, MNHN	France	/	2002	/	/	H1	/
AF034735(64)	Bone 1938-1296	France	Pointe de Calabre, Savoie	1900s	45.473	7.077	H1	/

Notes: Lat – latitude; Long – longitude; Cyt b hpt – cytochrome b haplotype; MHC allele – MHC DRB exon 2 allele. Caib-DRB*01 was detected in *Capra hircus* and deposit in GenBank with accession number AY706312.

Prvi vpogled v genetsko raznolikost alpskega kozoroga (*Capra ibex*) v Sloveniji

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Izvleček: V Evropi je bil alpski kozorog (*Capra ibex*) v 19. stoletju na robu izumrtja. Po tem času so se izvajali različni ukrepi za njegovo ohranjanje. V alpskem loku je bilo izvedenih več ponovnih naselitev, najprej z edino ohranjeno populacijo v narodnem parku Gran Paradiso v Italiji. Ozko grlo v preteklosti in dodatne ponovne naselitve so močno vplivale na genetski sklad populacije, tudi zaradi prisotnega genetskega zdrsa in parjenja v ožjem sorodstvu.V podporo znanstveno utemeljenim ukrepom ohranjanja so se za vse zasnovane populacije z izjemo Slovenije uporabljale tudi molekularne analize. Da bi razumeli, kako je ponovno naseljevanje in upravljanje vplivalo na genetsko variabilnost populacij v Sloveniji, smo analizirali nevtralni lokus (delni fragment mitohondrijskega citokroma b, mtDNA) in adaptivni poglavitni histokompatibilnostni kompleks (MHC DRB ekson 2) alpskega kozoroga iz dveh populacij (Julijske in Kamniško-Savinjske Alpe). Rezultati so pokazali, da sta obe populaciji genetsko zelo osiromašeni, saj nosita le en haplotip mtDNA in en funkcionalni alel za MHC DRB ekson 2, Caib-DRB*01. Zato so potrebni nadaljnji ukrepi za ohranjanje, vključno s ponovno naselitvijo živali iz populacij z večjo genetsko variabilnostjo. Vendar alpski kozorog v Sloveniji trenutno velja za tujerodno vrsto, kar zelo otežuje ukrepe za njegovo ohranitev in ogroža dolgoročno preživetje vrste.Znanstveniki in upravljavci populacij zato pozivajo politike/odločevalce, naj spremenijo status vrste v avtohtono in posledično omogočijo ponovno naselitev. Ti pozivi so podprti s predhodnimi arheološkimi podatki o obstoju kosti alpskega kozoroga v Julijskih Alpah in z dokazi o izraziti genetski osiromašenosti sedanjih populacij kozoroga, potrjenimi v tej študiji.

Ključne besede: *Capra ibex*; mitohondrijska DNA; MHC *DRB* exon 2; ponovna naselitev; upravljanje

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Slov Vet Res 2023; 60 (3): 115–172