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SCOPE

Iranian journal of Veterinary Science and Technology (IJVST) publishes important research advances in veterinary medicine and subject areas relevant to veterinary medicine including anatomy, physiology, pharmacology, bacteriology, biochemistry, biotechnology, food hygiene, public health, immunology, molecular biology, parasitology, pathology, virology, large and small animal medicine, poultry diseases, diseases of equine species, and aquaculture. Articles can comprise research findings in basic sciences, as well as applied veterinary findings and experimental studies and their impact on diagnosis, treatment, and prevention of diseases. IJVST publishes four kinds of manuscripts: Research Article, Review Article, Short Communication, and Case Report.

ON THE COVER

The artwork on the cover illustrates the entire globe as a fish suffering from several health problems. The mimic of the mouth of the fish shows gasping for fresh air and clean water, both in terrestrial locations and the oceans. Short fins seeming leaves over the body is the symbol of agriculture and forests. The footprint of an animal over the body, as the symbol of fish gills, not only represents the importance of animal health on the planet but also depicts the water- and carbon- footprints. The long fins are illustrations of kelps and coral reefs in the ocean, as an important part of marine life as well as shelters for aquatics. The disappearance of fish scales is an illustration of the extension of deserts in the world. The adverse global events, including anthropogenic conditions such as greenhouse gases emissions and massive withdrawal of freshwater resources along with natural disasters, e.g. El Niño and La Niña have caused major defects and destructions in the body of the compound word "One-health". A homunculus, as a symbol for ignorant humans, has got up very late after a long period of dormancy within the fish skull in a careless and irrespective attitude, after hearing the deafening sounds of gasping of the fish. She/he is watching the world through the fish eye, while canopying a hand over her/his eyes, to protect them from the burning sunshine as a symbol of global warming, to realize what the problem is. The artwork is created by Professor Arash Omidi, Department of Animal Health Management, Shiraz University, Shiraz, IRAN, and received further computerized illustrative effects by Dr. Behnaz Norouzi, DVM, DVSc. Paper by Dr. Kamran Sharifi (page 1) describes the required new roles, positions, and perspectives of food animal practitioners regarding the concepts of water footprint, carbon footprint, "One-health", and sustainability of animal husbandry and environment.

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TABLE OF CONTENTS

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Veterinary medicine and food animal practice in the era of footprints and "One-Health": a descriptive approach

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ABSTRACT

Our continually changing world has created new demands in society and has profoundly affected ecosystems, cultures, and professions. Ignoring the requirements and consequences of this ever-changing milieu could have devastating effects on all aspects of veterinary medicine. With the increasing global interconnections, several concepts have been created that should be addressed by the veterinary profession; otherwise, some instabilities will affect both the job and the society. In this article, these concepts will be critically analyzed and synthesized to portray an integrated perspective to address the necessities for the economic success of food animal practice, as well as describing the complicated role of veterinary medicine in the future. The first concept is the "evolving veterinary education", introduced by OIE in 2009, to address the new requirements of competent veterinarians who are able to respond and adapt to modern trading and business requirements. The second concept is "One-Health", which was introduced to address an integrated and all-inclusive perspective to health issues. All the specifications of this new concept are reflected in each letter of the word HEALTH (Humans, Ecosystems, Animals, Living Together, Harmoniously). The third concept is related to the "virtual water" theory, the total water consumed in the process of every activity, namely, the water footprint. It has been estimated that about 1000 and 15,500 liters of water are consumed in the process of production of a liter of milk and a kilogram of meat, respectively. Finally, the carbon footprint concept has been introduced to measure the total greenhouse gases emissions that enter into the atmosphere as carbon dioxide equivalent through individuals, events, organizations, services, places, products, and industries. The veterinary profession has a critical role and responsibility in the integration of the four abovementioned concepts.

Keywords

water footprint, carbon footprint, One-health, Food animal practice, sustainability of the environment

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Abbreviations

CO2-eq: CO2-equivalent
CP: Crude Protein
FAO: Food and Agriculture Organization of the United Nations
GHG: Greenhouse gases
GDP: Gross domestic product
GMP: Good manufacturing practices

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This article is dedicated to the memory of Larry Paul Thornburg, (1946-2019), a veterinary pathologist at the University of Missouri College of Veterinary Medicine, USA. I enjoyed a thoughtful journey by reading his nice insightful article, Four essential components of Veterinary education for the 21st century, in Journal of the American Veterinary Medical Association, 1992 Oct 15;201(8):1180-3, and translating it into Persian (Veterinarian Quarterly, 1998, 2:1, 29-34.).

Introduction

Animal husbandry has been started in the prehistoric era of human life, creating a paradigm shift in the lifestyle as well as the disease patterns of mankind. About 10,000 to 15,000 years ago when cattle were domesticated, the rinderpest virus, entered the human lives as the probable causal archevirus agent of several important diseases including, measles virus in human populations, and peste-des-petits ruminants virus in sheep flocks [1,2].

The first recorded documents pointing out to the veterinary practice backs to Babylonia's Laws of Hammurabi in around 2100 B.C. that include fees for treatment of a cow or an ass and penalty for malpractice. [3]. There is a considerable amount of literature and documents on the historical veterinary practice in ancient Iran that could probably be extended to all the Middle East [4].

The modern institutes of college-based veterinary practice were established in 1762, following the repeated attacks of rinderpest in Europe, after a decree by the council of State in France, led by Claude Bourgelat. The modern era for western veterinary medicine has been kick started in the Lyon Faculty of Veterinary Medicine, Lyon, France.

Between 1919 and 1935, several devastating attacks of rinderpest caused a huge cattle mortality in Iran, which eventually ruined agriculture as well, especially in the northern provinces. This repeated epidemics of the disease forced Iranians to establish modern western-based institutions such as the Faculty of Veterinary Medicine in Tehran University, the Iranian Veterinary Organization (IVO), and Razi Institute [4], in order to combat the consequences of rinderpest, at a period that lots of scientific endeavors should be devoted to have the Plowright tissue culture rinderpest virus developed in 1960 [5] . The famous pandemic of Influenza had caused heavy mortality around the world during 1918-1919 at that time. It has been reported that around 30,000 people succumbed to the so-called Spanish influenza just in Tehran, Iran [6]. The planet had to wait until 2005 to have the entire genome of 1918 H1N1 pandemic influenza been sequenced [7]

The role of veterinary medicine in Society

In the modern days, veterinary medicine is assumed to take responsibility for addressing health issues of all kinds of animals, however, at the beginning of modern veterinary medicine, the practice was mainly focused on diagnosing and treatment of livestock diseases. The owners of cattle herds and sheep/goats flocks, as well as native poultries, enjoyed largely from the innovations in the biological, pharmaceuticals, therapeutics, and surgical procedures in veterinary medicine. Sheep and goats production has been the cornerstone of the traditional subsistence livelihood, both in terms of nomadic and rural lifestyles, which is still current in developing and underdeveloped countries.

During the entire 20th century, fundamental progressions can be observed in the quality of lives of rural and nomadic people, after the development of the diagnostic preventive and therapeutic measures of control of devastating viral, bacterial and parasitic diseases, e.g. anthrax, foot-and-mouth disease (FMD), rinderpest, glanders, enterotoxemia, various animal poxes, agalactia, different types of pneumonia, Newcastle disease, parasitic diseases, etc.

The Control and monitoring foodborne diseases is another aspect of community expectations that should be addressed by veterinarians. It will start from the early time of decision making on the multifaceted therapeutic approach to diseased animals, in terms of the prediction of the outcome of diseases and withdrawal times of drugs, to slaughterhouse and carriage of the raw materials to the processing industries or house and restaurant kitchens. The contribution of veterinarians in tracing back the origin of the German outbreak of *Escherichia coli* O104:H4 associated with sprouts is an example of the fascinating contribution of veterinarians in ensuring food safety [8].

The need to keep companion and recreational animals has soared up since the 1960s in the US, a translocation of dogs and cats from farms to houses. Moreover, the transformation of lifestyles around the

Abbreviations-Cont'd

IOFC: Income Over Feed Cost
MDGs: Millennium Development Goals
N: Nitrogen
NPN: Non-Protein Nitrogen
OIE: World Organization for Animal Health (Office International des Epizooties)
PLF: Precision Livestock Farming
WHO: World Health Organization of the United Nations

world have made veterinarians an inseparable part of families' health, well-being, and happiness [3].

The inherent broad-based educational climate of veterinary medicine makes veterinarians, potentially eligible persons in many areas of research projects, including basic sciences, vaccinology, biotechnology, epidemiology, cancer research, and many other innumerable scientific domains. Such a broad contribution to the generation of biological and medical knowledge and science has an immeasurable impact on the sustainability of global society.

A changing world continually challenges veterinary medicine

An avalanche of social, technological, economic, and political trends continually challenges veterinary medicine. Lagging behind the critical events and ignorance of the upcoming changes might have serious consequences. From fifty veterinary colleges that had been established in the US during 1852-1924, thirty-nine schools ceased their operations when the internal combustion engines gradually replaced horses in the transport business. At that time, the veterinary profession had focused almost entirely on horses, paid very little attention to other livestock species. The introduction of coccidiostats shortly after World War II, when fed continuously at low levels, enabled the poultry industry to bypass veterinarians who bounded to the traditional concept of treating sick individual animals [3]. These historical experiences show that the veterinary profession should always be watchful of the impacts of social and global changes on the very existence of the profession.

Despite some miserable points in the history of the profession, veterinary medicine has had a spectacular cooperation and leadership in the development of modern food animal production from rural barns to huge industrial farms. Milk production per cow has steadily risen from less than a yearly average of 2 tonnes per cow to more than 8 tonnes in 2000 in the US [9]. In Denmark as an example of Western Europe, the milk production on an energy corrected milk basis (ECM) has increased from 1800 to 9000 kilograms in 2010 [10]. Field observations in the Iranian dairy cattle industry, which has largely set foot on the path of the US dairy cattle production model, show that primiparous heifers and multiparous cows with annual milk production of 11 and 13 tonnes, respectively, are not uncommon.

The same scenario has occurred in the poultry industry. The 100-days period for a broiler chicken to get a weight of 2 kg was decreased to 40 days in 2005. The feed efficiency (kg of feed to live weight ratio) was improved from 3 in 1960 to 1.7 in 2005. Annual eggs laid by a layer chicken in 1960 grew from 230 to 300 in 2005, while laying 5000 and 9000 eggs per tonnes of feed, respectively [11].

The problem of achieving concurrent high milk yield and reproductive performance in dairy herds can be viewed as a historical challenge that has been responded successfully. Milk yield in dairy cattle is highly correlated to reproductive indices. As previously stated, considerable progress in milk yield has been achieved from 1900 so far. The peak in the lactation curve is an important factor in determining the total milk productivity of typical dairy cattle. Based on the regression analysis of the Wisconsin milk yield data, Nordlund and cook (2004) reported that every pound (or kg) increase in milk production at peak means an extra 290-380 lbs (or kg) increase in rolling herd average milk yield [12]. Milk production of individual cows depend on the potential of the udder to produce milk, the supply of nutrients to the mammary glands, and farm management quality, as well [9].

It is widely accepted that with an incremental increase in milk yield, dairy cows may experience a state of negative energy balance (NEB) during the early lactation period, leading to losing some degrees of body condition score. It has been reported that some problems would raise, e.g. a severe body condition loss may increase the risk of pregnancy failure following the first artificial insemination [13]. Moreover, there is a negative correlation between the increase in serum non-esterified fatty acids (NEFA) and 3-hydroxy-butyrate (BHB) with pregnancy rate [14]. The decline in serum glucose levels is associated with a decrease in the rate of embryos that could develop to the blastocyst stage [15].

The reproductive indices profile seemed frustrating. In reviewing the trends in reproductive indices from 1970 to 2000, an increase in mean calving interval has been reported from 13.3 to 14.7 months, respectively. At the same period, the conception rate increased from 1.8 to 3, while the annual milk yield of dairy cows reached from 6400 to approximately 9000 kg [13]. There were claims that a negative relationship between milk production and reproductive performance does exist [17,18]. Some people advocated a longer calving interval of about 18 months to be appropriate for modern dairy cows [9], through genetic enhancement of persistent lactations and increasing animal welfare by managerial innovations [19].

Some researchers questioned the existence of a causal relationship between high milk yields and the decline in reproductive performance [20]. With a concurrent high performance in both milk production and reproductive indices, the share of the early period to the whole area under the curve of the lactation chart, compared with mid-and late- lactation

periods will increase, leading to a greater net income. With such a high performance in milk yield and reproduction, the maneuverability of dairy farms would increase, for example, in terms of financial resilience during economic crises. Moreover, with higher net incomes, the dairy industry would be able to invest in new technologies, e.g., earlier reliable pregnancy diagnosis [21], as well as new high-tech vaccines. According to computerized simulations, the economic value of every pregnancy was 278 $ compared with 555$ for a case of abortion, based on 2006 costs [22], indicating that a strategy of keeping both milk yield and reproductive measures at high levels is rational. Determining appropriate selection goals other than milk yield and reproductive indices, e.g. calf survival rate, metabolic diseases, and male fertility [23] should be considered as part of the concept of GMP, a criterion for ensuring that products are consistently produced and controlled according to quality standards within a system.

Multifaceted and multidisciplinary scientific cooperation among veterinary science and other scientific disciplines, e.g. basic sciences, animal sciences, agricultural economy, and agribusiness have resulted not only in the concurrent high-performance achievements in both milk yield and reproduction but also in other aspects of the dairy industry. Dozens of scientific trends can be traced in all aspects of food animal industries, for example, neonatal dairy calves, heifer rearing, genetics and breed improvements, hatcheries and pullet rearing that all are examples of an incremental intertwining process in all aspects of biology with animal welfare. The scientific trends just in colostrum studies [24, 25] are small grits in a sand hill.

Some lessons can be concluded from the aforementioned experiences: 1) Job opportunities for veterinarians that limit their practice to diagnosing diseases and treatment of single animals, at least in the food animal sector are incrementally fading. In the present highly dynamic situations, the art of diagnosis of diseases in a single animal remains crucial as an important part of the monitoring process of the behavior of diseases in the whole herd/flock; a trend of feeling the jungle not only through seeing the trees but by examining weeds. 2) Veterinarians should consider the economic and financial aspects during making decisions on a herd/flock basis. As an analogy, the role of veterinarians in food animal practice is increasingly getting more similarities to the industrial engineers in industrial plants, when production, management, and economics are highly integrated. 3) The old and emerging problems in the modern industries should be addressed through a multidisciplinary approach and a broad-based vision.

Sustainability of the food animal industry: the impact of diminishing return law

The progressive decline of rural livestock production in favor of increased urbanization caused an advantage towards the establishment of large food animal production facilities. A continual increase in average herd size with a concurrent decrease in the number of farms has been reported in the dairy industry in all developed countries [26]. It appears that the same scenario will be seen in developing and even underdeveloped countries, inevitably. It has been estimated that 15 percent of the total energy and 25 percent of dietary of the world, as well as essential micronutrients that are not easily obtained from plant-based foods, are supplied by livestock production [27]. Increasing the production to the maximum levels seems the best way to increase the monetary income as well as resilience during the economical/financial crises for typical farms. It has been shown, however, that optimum vs. maximum productions are not necessarily interchangeable concepts [9].

First described by economists, the diminishing return law is generally depicted by a curve that expresses the response to every unit of input on the horizontal axis, into efficiency output on the vertical axis. The omnipresence of this law can be seen not only in economics but also in many aspects of nature, e.g. bacterial growth rate curve, clinical medicine [28], pharmacology [29], as well as the increase in milk yield in response to increasing the proportion of concentrates to forages in dairy cattle ration. The latter could even probably be used to describe the situations leading to subacute ruminal acidosis (SARA) in dairy cattle. Based on this law, additional inputs produce smaller and smaller outputs to a system from a certain point, and the efficiency curve trend goes to get flattened. Thus, using diets maximizing milk production may not be the most profitable policy [9]. For a long time, the board of senior consultants of dairy farms was comprised of at least a nutrition advisor (either a nutritionist or a veterinarian), a theriogenologist or an expert veterinarian for reproductive issues, a veterinarian (may also be a specialist to manage herd health issues) and a geneticist to improve the genetic merit of the herd/flock. In the current situations, an agricultural economist is highly needed as well, to find the best points of inputs for optimum production efficiency and ensuring sustainability of the herds/flocks and economical resilience of the industry.

The need to dynamically updating veterinary curricula and educational climate

Thornurg (1992) published an article with the fascinating title “Four essential components of vet-

erinary medicine for the 21st century" [30]. He critically reviewed the points of weakness of veterinary education and proposed the four components that should be included in veterinary curricula as follows: a broad-based education, nurturing critical thinking abilities in the students, induction of the diagnostic competency in graduates, and institutionalizing the enthusiasm of lifelong self-education in veterinarians. At that time, he was very concerned about how the students and veterinarians can cope with the so-called information explosion that has sky-rocketed these days. According to him, the veterinary curriculum in the US has been extensively and critically reviewed by the American Veterinary Medical association in 1931, which preceded the report of the Pew National Veterinary program by Pritchard in 1989 [3]. From that time, a considerable amount of articles and valuable innovations and modifications have been introduced in veterinary curricula, mostly in Western countries.

A comprehensive special issue entitled "Veterinary education for global animal and public health" has been published by Revue Scientifique et Technique, the journal of OIE (number, 2, volume 28, 2009). The collaborations of deans and associate deans of veterinary medicine Faculties in this issue were considerable. In 2009, OIE held a conference under the title of "evolving veterinary education" to address the specifications of the veterinarians to deal with the new expectations of a highly dynamic world as well as the requirements of globalization, keeping pace with the basic requirements in terms of the capacity of the public and private components of veterinary services in the field of animal disease surveillance and control in a globally broad-based perspective [31].

The current situations and expectations of the world societies are highly dynamic. During a tripartite meeting in 2010, WHO, FAO, and OIE committed to coordinate their efforts to solve the overlapping issues of the three organizations. The last session was held in 2017, determining three major subjects to deal with simultaneously, including rabies, zoonotic influenza, and resistance to antimicrobials. A dozen of other subsidiary issues were also discussed [32]. At that time, nobody had any idea about the pandemic of COVID-19 in the near future.

There are numerous compelling evidences in favor of the critical role of veterinary medicine in a changing world. Global warming facilitates the spread of infectious agents to previously clean countries. The spread of bluetongue virus [33] and Lumpy skin disease [34] from Africa to Europe and then, the Eastern Mediterranean basin, respectively, causing major economic losses can be taken into account for emphasizing as an urgent necessity that the surveillance and control of animal health issues should be reviewed in order to be compatible with the requirements of a new era of interplay between infectious diseases and the planet.

The poisoning by melamine is another historical experience showing the crucial role of veterinarians in the discovery of health hazards to society. Melamine (1,3,5-triazine-2,4,6-triamine. C3H6N6, molecular weight=126) an industrially synthetic chemical, contains a high level of N (66.6%). The deliberate addition of small amounts of melamine will mistakenly lead to a considerable rise in CP contents of feedstuffs and food products. As part of the series of Proximate analysis that was designed to indirectly estimate, not precisely measure, the nutrients and chemical composition of feed/food samples through relatively and economically justifiable prices, the N content (%) of a typical sample are estimated by the Kjeldahl method at first step. There are specific coefficients for a wide variety of feedstuffs, however, 6.25 is generally accepted as the mean of coefficients and used as something like a wildcard. The multiplication of the N content of melamine (66.6%) by 6.25 leads to 416.67% of the CP content of melamine. In other words, the addition of 100 grams of melamine to a product, will increase the CP content of the final mix by about 416.67 grams. Borrowed from computer literature, it is a bug or a security hole in the Kjeldahl method, which is unable to differentiate between NPN and true protein sources, as the suppliers of N in the feed/food samples.

A causal relationship was made between some specific pet foods to nephrotoxic cases in 2004 and 2007 that finally the phenomenon was known as melamine-induced nephrotoxicity [35]. These kinds of food adulteration gave rise to public health concerns [36], which has been traced back to imported and domestic products in Iran, as well [37]. These reports show that even those sectors of veterinary medicine that are not professionally related to food animal practice and food supplying sectors should have astute, vigilance, and preparedness in addressing the health status and expectations of the society originated from edible products of food producing animals.

Thornburg (1992) expressed his worries about the shortcomings of workaholic nonflexible veterinary curricula that were suffocated by an avalanche of discipline-based courses in educating competent veterinarians to deal with emerging and re-emerging problems in the society. Revision of the veterinary education is the starting point and has a pivotal role for the whole profession to play a highly efficient role in society; however, this task has received minor attention at least in the developing countries, those that urgently need a renewal of their veterinary curricula.

One health and the leading role of veterinary medicine in the community

It is known from ancient times that the health status of human beings, animals, plants, and environment are highly interrelated. There are reports suggesting that the ancient Egyptian disasters might be triggered by ecological issues [38]. Robert Matthews (1999) through a striking subtitle in his nice article has pointed out the fine interrelationships among the aforementioned constituents of the concept of "One Health" as follows:

Through an arrogant or ignorant disregard for ecological complexities, ceaseless human encroachment on nature can unleash a terrible new threat of killer diseases carried by microbes that have long lain undisturbed [39].

Veterinarians were tirelessly warning the outcomes of the neglects to a unified perspective to human and animal health from the start of the modern veterinary medicine and science in Iran, but frequently received feedbacks were implicating that the word health was just limited to humans. As a result, a frustrating numbness was displayed by disciplines that were inherently responsible to participate in a comprehensive approach to health hazards. Just due to the recent devastating emerging and re-emerging diseases, however, obliged the community to reconsider the health-related concepts critically.

Part of the problem in the delay to realize the importance of "one health" may be due to this fact that there are different perspectives toward health issues. The letters in the HEALTH acronym denote the inherent integrated health components: Humans, Ecosystems, Animals, Living, Together, Harmoniously [40].This fundamental perspective leading to a harmonious comprehensive approach to health concept in a globally accepted interpretation should be supported by scaffolds are to be constructed by an integrated education and interdisciplinary cooperations [41,42].

Reminding the issues discussed on the difference between optimal and maximal production levels, it is noteworthy that targeting the maximum economic income might have adverse environmental impacts. On a national approach in dairy cattle industry in the US, It has been estimated that choosing the IOFC criterion versus maximum efficiency of N means increasing the CP contents of a typical dairy cow producing 35 kg milk /day from 14.9% to 18%, respectively. The inevitable outcome of such an increase in the CP content of the rations other than an increase in net monetary in-

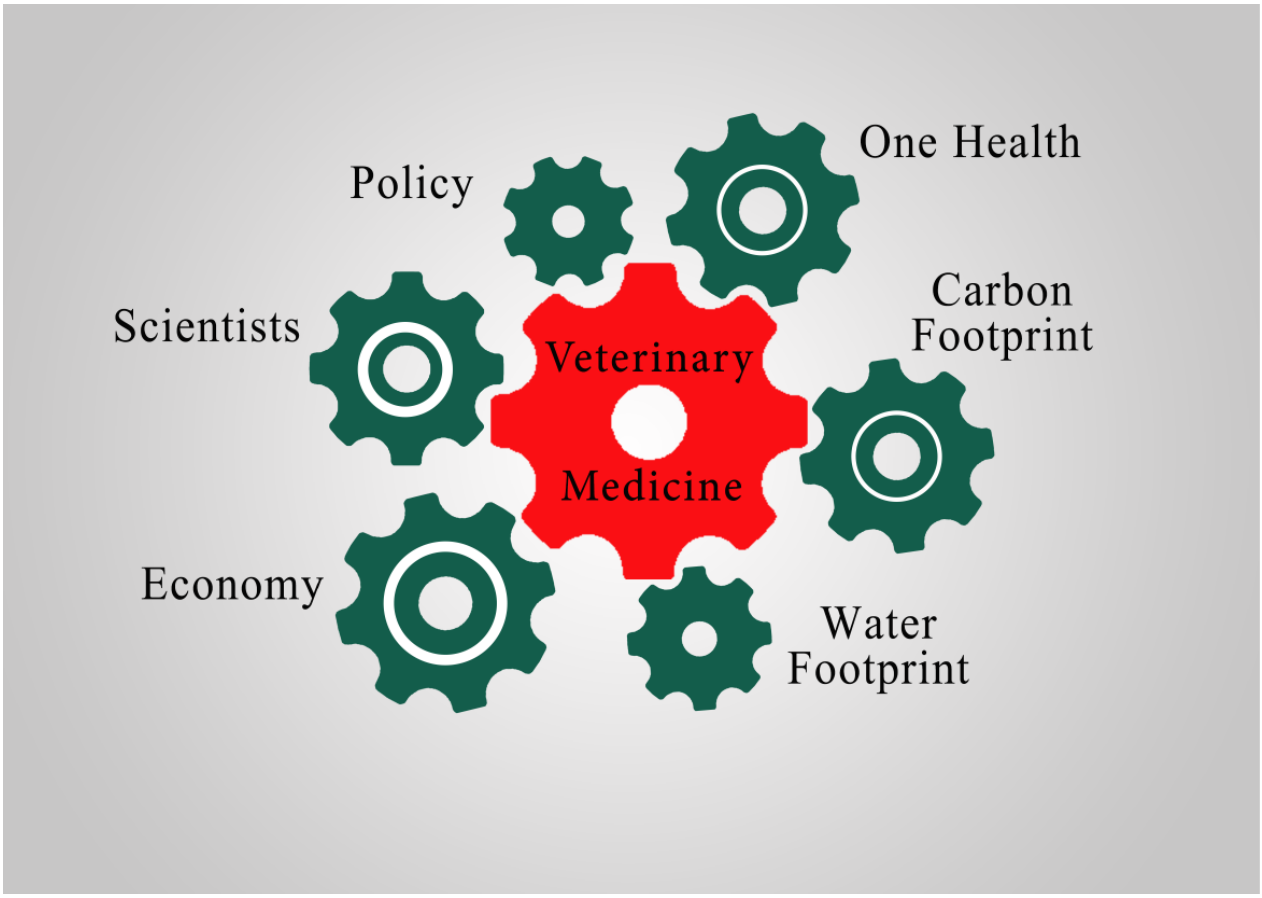

Figure 1.
The integration of different subjects to be addressed by veterinarians. Adapted from King, 2009 (45).

come is the excretion of an extra 150,000 metric tons of N into the environment [9,43]. If some regulatory laws are to be assigned for industries to oblige them to compensate for the adverse environmental impacts of their activities, this may submit an impulse to encourage the industry to pursue more environment-friendly strategies rather than focusing merely on maximum monetary income.

The same scenario, in terms of energy, might be true for the counterpart of IOFC in the poultry industry, the feed conversion ratio. The interaction of genetic changes and the trends seen in both the energy use efficiency and the heat production rate has been critically reviewed [44]. These examples clearly show that food animal industries should consider both the economic and environmental aspects of production, simultaneously; being economically profitable and environmentally justifiable. The interrelated areas of One-health that should be kept working together harmonically and systematically by veterinarians as one of their major responsibilities have been depicted in Figure 1.

Water footprint-virtual water-embedded water concept in food animal practice

The water footprint of a product is the volume of freshwater used to produce the product, measured over the full supply chain. It consists of the blue water footprint resources (surface and groundwater), green water footprint (rainwater insofar as it does not become run-off), and the grey water footprint (the volume of freshwater that is required to absorb the load of pollutants given natural background concentrations and existing ambient quality standards. It may be frustrating for the food-producing animal industries to realize that for the production of each kg of beef meat, milk, cheese, and chicken meat about 15,500, 1000, 5000, and 3900 liters of water are consumed throughout the full production line, respectively [46]. With the introduction of the concept of virtual water, the sustainability of food animal industries, in terms of economic and environmental issues will be highly complicated, especially in areas suffering from chronic drought or lack of sufficient renewable water resources.

The problem of water resources not only can be evaluated as a hot topic but as a dangerous situation for water security. It has been stated that about 74 cubic kilometers of groundwater in Iran has been withdrawn during 2002-2015 [47], meaning a state of water bankruptcy. The continuous overexploitation of water resources in Iran has reached hazardous levels, in terms of water security as well as the danger of increased water salinity. The cumulative withdrawal of nonrenewable groundwater in Iran from 1965 soared to 1.33×10^{11} m^3 in 2019 [48].

The industrial and rural dairy cattle and small ruminant production have been under the pressure of chronic drought situations in terms of supplying economically justifiable forages. The minimum recommended levels of forages should be met to ensure the rumen health status and efficient use of the concentrate ingredients of the ration in ruminant animals. The prices of concentrate and forage in the UK are reported to be about 160£ and 60£, respectively on a dry matter basis with a ratio of 2.67 in 2010 [49]. The price of different forages have chronically been equal to or higher than concentrates in Iran. The relatively higher prices of forages compared to concentrates can be accounted as a major constrain to the sustainability of the ruminant sector of food animal production industry. On the other hand, hidden subsidies paid to fuel and electricity consumption in oil-rich countries, if not managed on virtual water concept, would exacerbate the environmental unsustainability, by preventing the real value of water being taken into account. To match the personal, social, and environmental interests as much as possible through the action of the so-called Adam Smith's invisible hand, high levels of transparency are needed.

With the current drought conditions in Iran which are unprecedented in the last 50 years, the import of forages like alfalfa hay or alfalfa meal, is an urgent tactical intervention, to save the herd/flock owners from financial bankruptcy as well as ameliorating the pressure on the rangelands. It has been reported that the water footprint of alfalfa hay in Argentina ranged from about 728 to 881 $m^3\ t^{-1}$ as virtual or embedded water [50]. In these critical periods, the temptation to irrigate vegetables for human consumption and/or forages for ruminant feeding by unrefined wastewater should be considered as a serious criminal health threat to humans, animals, agriculture and the environment and planet, altogether. Strict regulatory laws and penalties should be enacted to restrain such a terrible villainy. Facilitation of the international trade of virtual water, e.g. as international import and export of vegetables and forages can be considered as a strategy in order to dissuade using wastewater for irrigation. Virtual water trade potentially is helpful to mitigate the global water crisis, in addition to virtual water flow for agricultural products [51].

Carbon footprint and food animal production

While not as locally sensible as virtual water, the carbon footprint is considered an important issue from a global perspective. Greenhouse gases, including methane, nitrous oxide, and carbon dioxide are emitted into the atmosphere by dairy production. The global dairy sector contributes 4.0 percent to the total

global anthropogenic GHG emissions [52].

It should be noted that deforestation might be accounted as part of the prerequisites for the initial phase of the establishment process of dairy farms in many locations in the world; a major contribution to the GHG emissions phenomenon. The share of pig and chicken in the annual GHG emissions are 669 million tonnes and 606 million tonnes CO_2-eq, respectively [53].

The paradox of increasing demand for food from animal sources, and the urgent need to decrease GHG emissions at the same time, should be addressed by the interrelated disciplines involved in food animal practice. As much as possible, deforestation should be considered as a major obstacle in establishing new food animal facilities, monitored by regulatory laws.

The importance of politics

A mutual interrelationships among scientists and politicians are needed. It should be taken into consideration that many decisions may have environmental side effects that should be scrutinized by both parties. The strategies like self-sufficiency on high water demanding products, e.g. wheat (1300 liter/kg) or milk (1000 liter/kg) [46] in countries with scarce water resources could have devastating outcomes. Moreover, the apparently water-conserving activities, such as building dams in arid and semi-arid countries may have a backfire outcome. The drying out of the majority of the internal lakes in Iran should be critically evaluated because dried-out lands would had direct negative effects on the food animal practice and potential job-creativity of animal husbandry. The dams at the upstreams of Tigris and Euphrates in Turkey may result in the drying out of the downstreams at the south Iraq, leading to terrible sand storms in the region. The southeastern Sistan and Baluchestan province of Iran as the downstream, is suffering from the dam(s) at the upstream of Hirmand (Helmand) river in Afghanistan, to the point that Hamoon lake has been dried out for many years, ruining fishing, fisheries and animal husbandry activities in the region as well as the livelihoods, causing considerable immigration of the native people. It is advisable to review the dam construction policy or craziness, at least in the Middle East, where a proinflammatory status is present for further conflicts on water. At the present time a dam removal debate is current in scientific communities in the US [54].

The approval of laws and major decisions on country/regional/global scales should always be done under the umbrella of sustainability of the environment. An international common sense is needed to exempt health and environmental sustainability issues from domestic and/or international political conflicts, for example, sanctions.

Conclusion

Setting foot on the path of development is not a straightforward process. Lots of information, disciplines, and specialties should come together, to increase the precision of decisions and accuracy of actions. Agribusiness, defined as the food sector of economy, should be considered as an important part of the domestic as well as international political stability. Every wrong step may not only result into environmental adverse effects, but social and economic crises could potentially spin out of control (55). Veterinary medicine has a pivotal role in integrating health issues, economy and environmental sustainability, decreasing poverty, ensuring security, and increasing the welfare of humans, animals, and the planet in the era of footprints and "One-Health". It should be taken into consideration that the starting point to address all these highly complicated tasks is providing high quality, broad-based education by all disciplines, and in this case by veterinary medical schools and faculties. The critical role of veterinary science and veterinary medicine in the substantiation of "global vision and local action" has been depicted in Figure 2.

Veterinarians are among the first responders in the frontline of the battle against threatening global issues that are always ready to escalate to worrying dimensions, including population growth, economic stagnation, environmental stress, increased prices of food animals and products that are now flowing in international markets, emerging and re-emerging infectious and exotic diseases, bioterrorism, public health hazards, etc. [56].The engagement of scientists and veterinarians in strengthening biosecurity systems is of paramount importance to ensure resilience and sustainability [57].

Veterinary medicine should play its leadership role as one of the most talented disciplines in global and environmental issues [56]. One of the key interconnecting rings in the armor that the modern world should wear against the threatening crises is veterinary medicine, which is designed to achieve economic promotion as well as stability, sustainability and salvage of the environment and planet.

A major part of the successful achievements and promotion in food animal industries is due to the artistic integration of animal welfare concepts and criteria, as an important factor both from production efficiency and disease prevention perspectives, into the whole practice of food animal production medicine. The concept of PLF, as a potential strategy to mitigate environmental risks of livestock farming [58] is an-

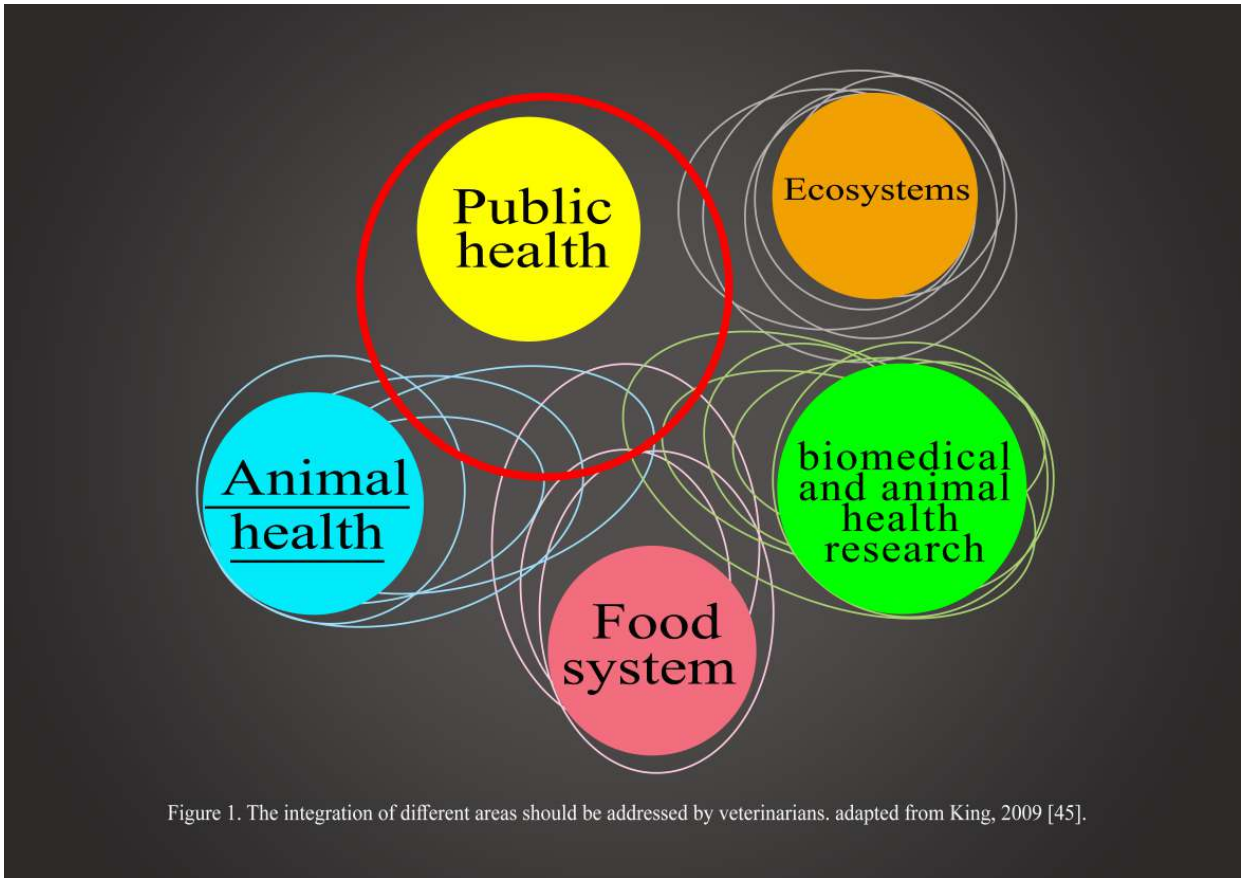

Figure 2.
An illustration depicting the central role of veterinary medicine in informative connection and leadership between policymakers, economists, and various scientific disciplines to address On-Health, water- and carbon-footprints in an ever-changing world.

other step in the evolution of livestock production industry. All these concepts makes the emergence from rural activities to industries feasible; from piggeries to Muyuand foods hog enterprise (Nanyang, China); from backyard poultry production to Egg city in California, from barns to Mudanjiang City Mega Farm (Heilongjiang, China). But it is not yet enough.

Future directions

As previously described, virtual water is an important determinant in the sustainability of the environment and the development of the community. It is true that high virtual water is demanded in the process of the production of meat, milk, eggs and chicken meat, the interconnection between high and relatively low demanding virtual water industries, would potentially provide more efficient less destructive use of the ever-limited resources. Just as an example, the share of the tourism industry in GDPs of countries is getting more attention globally. It is reported that tourism accounts for more than 10% of Spain's GDP and the total water consumption of the residents and non-resident tourists equals 9.985 km^3. The required blue and green water consumption to meet the demand of non-residents (foreign tourists) in Spain is 3.737 km^3 and the requirements from Spanish resources just equals 1.570 km^3, while consumption of Spanish blue water resources is 0.885 km^3. The latter is politically controlled, which is the subject of public debate as well, in Spain [59].

It seems that it is rational for the food animal industries to follow new paths in order to be integrated into new markets as well as promote their roles from an agro-alimentary activity to agro-alimentos-industrial levels, such as tourism industry, which potentially provides higher payments for a defined amount of nutrients in terms of carbohydrates and proteins supplied by the native residents of a country, provided that the quality, esthetic and organoleptic measures of the final product on the plate in front of the enthusiastic tourists seeking new experiences are guaranteed. For the countries that have all the components of a tourism destination, "sea/sun/sand", a multisectoral relevance among different disciplines, e.g. policy, economics, agriculture, veterinary medicine, and tourism should be constructed. The final interplay should be reflected in the increased GDP. In the modern world, veterinary medicine should follow the approaches to highlight its share in GDP, by taking part in "one-health" and increasing efficiency, based on the issues raised by the era of footprints and "One-Health". Med-

ical tourism is another example of increasing the income that could potentially be reflected in the promotion of the economic and health status of the tourism destinations that are providing or potentially are able to supply medical-services as well.

Other similar collaborative remedies should be sought by veterinarians as well as other disciplines to decrease the current intolerable pressure on water resources, land and sustainability of environment and planet through the reincarnation of environment-friendly industrial soul into the bodies of agriculture, agribusiness, human and animal health. The extra income from these innovations could be invested in education, seeking innovations and new technologies, research and/or compensation of the harms insulted to the environment by the industry. Veterinarians should be appreciated as vigilant observers of a broad planet issues; ensuring sustainable production. Political decisions, on domestic or international scales, which may potentially affect environment should be critically monitored by a variety of scientists, including veterinarians. It is tempting to deforest a jungle to establish dairy or beef cattle rearing farms for both the investors and peoples that are threatened by a damping economy, which ruins the versatile job opportunities. The scandal in Brazil, "Bolsonaro's treatment of the amazon" is condemned by celebrities and world leaders [60], is a prominent example that should have received prompt and on time reactions by various scientists; especially veterinarians should always be ready to swim against the tide, be prepared to show rapid reaction, in addition to publishing scientific articles that will be inherently submitted with a considerable delay, when the public has forgotten the relevant crisis. Without a sustainable economy and society, as well as reliable job opportunities, resurgence of the instability conditions may have destructive effects on the sustainability of the environment. "Bolsonaro's treatment of the amazon" in Brazil has had roots in the past; chronic pathologic sociological states that had been described as land-hungry [61], which has been led by fanning the flames of greed [62] were present for decades before, as hidden fire under the ashes. The same scenario would be possible to be occurred everywhere, as well as Iran. It has been warned that shortcomings in relevant laws oriented toward jungle preservation, would invariably result into degradation of forests in Iran [63].

Several currencies should be managed rationally in order to address health and economics issues, from ATP in the intracellular biochemical processes to strong currencies in the global market. Veterinary Medicine and veterinarians are among those few disciplines that can have an influential vision on both sides; from the depth of the cells to the highlands of health, environment, economy, technology, engineering and political issues, deserving their global leadership role, if environmental commons are to be managed sustainably, not selfishly base on self-interest beneficiaries, because many human societies might not reach the post-industrialization stage in the near future [64]. The veterinary education has a pivotal role in the keeping lots of concepts in a united perspective, e.g. water footprint, carbon foot, environment sustainability, GDP, PLF as well as providing efficient and meaningful interconnections among major institutions like OIE, WHO and FAO together, in addition to scientists, politicians and governments who are supposed to be highly committed to MDGs [65]. These goals were signed and declared in September, 2000, programmed to be achieved till 2015. At the present time, it is superseded by a more comprehensive renewed targets named Sustainable Development Goals [66]. The critical role of veterinarians in the accomplishment of the latter is undeniable.

Authors' Contributions

The author has written, read and approved the first and final drafts of the manuscript.

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Conflict of interest

The author has nothing to disclose, however, was highly worried about the arrogant and ignorant disregard taking place toward the ecology, destiny of the people and planet during the writing of this manuscript.

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Detection of mutant infectious bronchitis viruses of GI-23 lineage from commercial chicken flocks in Khorasan Razavi province, Iran in 2019

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ABSTRACT

Infectious bronchitis (IB), caused by infectious bronchitis virus (IBV), is one of the most important respiratory diseases in poultry. The implementation of preventive measures, including vaccination and biosecurity, is necessary for controlling the disease. To maintain biosecurity, it is important to identify the entry route of new viruses into a region and characterizing markers such as unique mutations that make viruses traceable. During a genotyping study for IBV infected commercial chicken flocks in Khorasan Razavi province, 11 viruses from 11 broiler and layer chicken flocks were detected in different cities by PCR. Sequencing of the S1 partial gene followed by phylogenetic analysis showed that eight viruses can be classified in GI-23 lineage (Is-Variant2), two viruses are classified in GI-1 lineage (Mass), and one virus is classified in GI-12 lineage (793B). Although detected viruses of GI-23 lineage are originated from Iran, seven viruses have synonymous (T954C and G1056A) and non-synonymous (C797T) mutations that have not been previously reported. It was found that the new genetic changes in Iranian IBVs of GI-23 lineage occurred in two different regions in Khorasan Razavi. In conclusion, this study indicates that the high prevalence of GI-23 lineage viruses in Iran may enhance the chance of virus mutations and the emergence of new viral strains, so effective vaccination and biosecurity measures are required to control the virus spread.

Keywords

IBV, genotyping, GI-23 lineage, mutation, Iran

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Abbreviations

IBV: infectious bronchitis virus
IB: infectious bronchitis
PCR: polymerase chain reaction
GI: genotype I
Mass: Massachusetts

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Introduction

Infectious bronchitis virus (IBV) causes a respiratory disease called avian infectious bronchitis (IB) that was first reported in the 1930s in the United States [1]. IB is highly contagious and causes severe economic losses in the poultry industry worldwide [2]. All strains of IBV can replicate in the respiratory tract of birds and cause respiratory diseases. Some IBV strains can also target epithelial cells in the oviducts and kidneys and may cause a significant reduction in egg production, nephritis, and mortality [2]. The pathogenicity of different strains of IBV can be categorized from mild respiratory involvement to severe kidney disease [3].

IBV has a single-stranded, positive-sense, RNA genome approximately 27 kb in length. The 3' end of the genome encodes four non-structural proteins, including 3a, 3b, 5a, and 5b, as well as four structural proteins, including the glycoprotein spike (S), envelope (E), membrane (M), and nucleocapsid (N). The 5' end of the genome encodes two polypeptides (1a and 1ab) that are required for RNA amplification [4]. Genetic variation in IBV can occur following recombination or mutations such as deletion, insertion, and substitution during virus replication [2].

The high rate of mutation in the genome of coronaviruses is associated with the poor ability of enzymatic correction of mutations (3' to 5' exoribonuclease activity) during replication [5]. The spike protein is composed of about 1145 amino acids and is cleaved into two subunits, S1 and S2, following post-translational modifications [6]. The S1 protein determines the virus serotype and contains the neutralizing epitopes. Considering the IBV genetic variation and the highest diversity of the S1 gene, the emergence of new serotypes and genotypes is expectable [6]. The high rate of changes has led to the emergence of new IBV genotypes in different parts of the world [7]. According to the latest IBV genotyping method by using the S1 gene, six genotypes are defined that comprise 32 distinct viral lineages and some inter-lineage recombinants [7]. Genotype I (GI) includes 27 lineages and each of the remaining five genotypes contains 1 lineage. Lineages such as GI-1 (Mass type), GI-13 (793B type), GI-19 (LX4 or QX), GI-16 (Q1), GI-21 (Italy02), and GI-23 (Israel variant 2), have been reported in several continents, countries, or regions, while other lineages are limited to specific regions of the world [7].

The first report of IBV infection in chicken flocks in Iran was published in 1994 [8]. Since then, the IBV prevalence in Iran has been reported by several studies [9-11]. In the latest study in Khorasan Razavi province in 2016, GI-23 lineage viruses were common and had a 100% genetic similarity to other Iranian IBVs [12]. GI-23 lineage viruses have also the highest prevalence in Iran [9, 10].

As in previous studies reported from Khorasan Razavi and Iran [9, 10, 12], we found that GI-23 lineage viruses are prevalent in Khorasan Razavi, with the difference that Is-Variant2 viruses detected in this study have point mutations that distinguish them from previously reported viruses. This finding emphasizes that effective vaccination and biosecurity programs are needed to prevent the emergence and spread of new IBV strains. The viruses detected in this study will be traceable due to specific mutations and will make it possible to track outbreak routes of a virus in different regions.

Results

IBV detected in 11 commercial chicken flocks by PCR and sequencing

Among the 15 IB suspected commercial broiler and layer chicken flocks in Khorasan Razavi, 11 flocks belonging to the cities of Mashhad (5 flocks), Torbat Heydariyeh (2 flocks), Quchan (1 flock), Gonabad (1 flock), Kadkan (1 flock), and Chenaran (1 flock) were IBV positive.

Prevalence of GI-23 lineage viruses with synonymous and non-synonymous mutations in the Khorasan Razavi province

The partial sequences of the S1 gene of positive samples were submitted to GenBank, and accession numbers MW366335 to MW366345 were assigned to the 11 detected viruses. The nucleotide sequences of these viruses, along with reference strains and some Iranian IBVs were used for phylogenetic analysis. Out of 11 viruses, eight viruses detected in Mashhad, Torbat Heydariyeh, Gonabad, Quchan, Kadkan, and Chenaran were classified as GI-23 lineage (Israel Variant 2). From three strains identified in Mashhad two were classified as GI-1 lineage (Mass type) and one as GI-13 lineage (793B type) (Figure 1).

Four GI-23 viruses identified in Mashhad (MW366340 and MW366344), Chenaran (MW366345), and Quchan (MW366342) formed a distinct subbranch (60% bootstrap support) and are 100% similar in nucleic acid sequence (Figures 1 and 2). These DNA sequences BLASTed against GenBank (December 2020). The highest similarity (99.38%) was related to 8 viruses. These viruses include IBV/Chicken/Iran/IS1494-like/MRB02/2016 (MG013973), IBV/Chicken/Iran/IS1494-like/MRB01/2016 (MG013972), Iran/Bu/Variant2/SH1229.7/14 (KX578827), Iran/variant 2/H272/12 (KP310024), SEMNAN/18/2018 (MN794044), SEMNAN/10/2018 (MN794036), SEM-

NAN/5/2018 (MN794031) and SEMNAN/4/2018 (MN794030), all detected in Iran during the 2010s. Synonymous point mutations including T954C and G1056A were identified in the S1 gene compared to the previously reported Iranian IBVs.

The three viruses identified in the cities of Torbat Heydarieh (MW366335 and MW366336) and Gonabad (MW366341) were also classified in the GI-23 lineage and formed a distinct subbranch (65% bootstrap support) (Figures 1 and 2). These viruses are 100% similar in nucleic acid sequence. Following BLASTing the nucleotide sequences against GenBank (December 2020), the highest similarity (99.7%) was related to 11 viruses. These viruses include IBV/Chicken/Iran/IS1494-like/MRB02/2016 (MG013973), IBV/Chicken/Iran/IS1494-like/MRB01/2016 (MG013972), Iran/Bu/Variant2/SH1450.19/15 (KX578834), Iran/Bu/Variant2/SH1450.5/15 (KX578832), Iran/Bu/Variant2/SH1229.7/14 (KX578827), Iran/variant 2/H272/12 (KP310024), Iran/Variant 2/H100/11 (KP310023), SEMNAN/18/2018 (MN794044), SEMNAN/10/2018 (MN794036), SEMNAN/5/2018 (MN794031) and SEMNAN/4/2018 (MN794030), all identified in Iran during the 2010s. A non-synonymous point mutation C797T that results in an amino acid exchange of Thr266Met was identified in the S1 gene compared to the previously reported Iranian IBVs.

The virus (MW366343) was identified in Kadkan city was clustered along with other Iranian GI-23 lineage viruses (Figure 1). This virus shows 100% similarity with the most previously reported Iranian IBVs of the GI-23 lineage.

The virus (MW366338) detected in Mashhad was classified in GI-11 (Figure 1). Nucleotide analysis showed that this virus is 100% similar to the 793B vaccine virus.

Based on the phylogenetic tree, two IBVs which were identified in Mashhad (MW366337 and MW366339) are classified as GI-1. However, these two viruses, with 100% similarity, formed a distinct subbranch (67% bootstrap support) (Figure 1). The nucleotide sequences BLASTed against GenBank (December 2020), the highest similarity (100%) was related to 3 viruses including Iran/Mass/H350/12 (KP310052), ck/CH/LJL/121059/2012 (KJ425509), and China/vaccine strain/HK/2004 (AY761141). Analysis of nucleotide similarities showed that the nucleotide sequences of these two viruses are 99.68%, 99.36%, and 98.73% similar to Ma5, H120, and H52 vaccine viruses, respectively; however, the protein sequences are 100% similar.

Phylogenetic analysis based on amino acid sequences of GI-23 lineage viruses

The deduced amino acid sequences of GI-23 lineage viruses corresponding to partial S1 protein used for the phylogenetic tree construction (Figure 3). The 5 viruses detected in Mashhad (MW366340 and MW366344), Chenaran (MW366345), Quchan (MW366342), and Kadkan (MW366343) were clustered along with other GI-23 lineage viruses reported from Iran. The protein sequences of these 5 viruses are 100% similar to the most Iranian GI-23 viruses previously reported. The amino acid sequences BLASTed against GenBank (December 2020). The highest similarity (100%) was related to 4 viruses including SEMNAN/3/2018 (QLF98578), Iran/Bu/Variant2 /SH1229.7/14 (ART85681), SEMNAN/10/2018 (QLF98585), and Iran/variant 2/H272/12 (AKH60829).

The viruses detected in Torbat Heydarieh (MW366335 and MW366336) and Gonabad (MW366341) are 100% similar in amino acid sequence and form a distinct subbranch (65% bootstrap support) (Figure 3). For further investigation, the amino acid sequences BLASTed against GenBank (December 2020). The most similar viruses were 99.1% similar and included 7 viruses Iran/Bu/Variant2/SH1450.5/15 (ART85686), Iran/Bu/Variant2/SH1450.19/15 (ART85688), SEMNAN/3/2018 (QLF98578), Iran/Variant 2/H100/11 (AKH60828), Iran/Bu/Variant2/SH1229.7/14 (ART85681), SEMNAN/10/2018 (QLF98585) and Iran/variant 2/H272/12 (AKH60829).

Discussion

The GI-23 lineage represents a cluster of unique wild-type viruses that are geographically limited to the Middle East. Strains belonging to this lineage have been identified in Israel since 1998 and are still circulating in the region [10, 13]. Some of these viruses like Is-Variant2 have become prevalent and affect the respiratory and renal systems [14]. Studies in recent years have shown that variant 2 viruses are common in Iran [9, 10].

Among the GI-23 lineage IBVs identified in this study, the virus detected in the city of Kadkan (MW366343) has 100% nucleotide similarity with the viruses reported in the Khorasan Razavi province in 2016 [12] and other GI-23 lineage viruses previously reported in Iran [9]. Accordingly, this virus is currently present from the previous outbreaks of GI-23 lineage viruses in Iran.

The other seven GI-23 lineage viruses of this study have point mutations different from other viruses reported in GenBank. These viruses formed two distinct subbranches, and each subbranch contained similar viruses that were detected in adjacent cities (Figures 1 and 2).

Nucleotide BLAST showed that there are no re-

ported viruses with 100% similarity to GI-23 lineage viruses detected in Mashhad (MW366340 and MW366344), Chenaran (MW366345), and Quchan (MW366342). However, the closest sequences (8 viruses) with 99.38% similarity have been reported from Iran during the 2010s, and all differed from the viruses of this study in two bases, 954 and 1056. Nevertheless, the amino acid sequence is 100% similar to the Iranian GI-23 viruses. Accordingly, unique synonymous point mutations make the virus traceable in future studies. It should be noted that the virus had the chance to spread to adjacent cities (Figure 2).

The other GI-23 lineage IBVs detected in Torbat Heydarieh (MW366335 and MW366336) and Gonabad (MW366341) are 100% similar (Figures 1 and 2). Nucleotide and protein BLAST showed that the sequence with 100% similarity was not registered in GenBank. The most similar sequences to these viruses have been reported from Iran; so, these viruses also originated in Iran. However, a non-synonymous point mutation (C797T) has been created in the detected viruses compared to the previously reported ones.

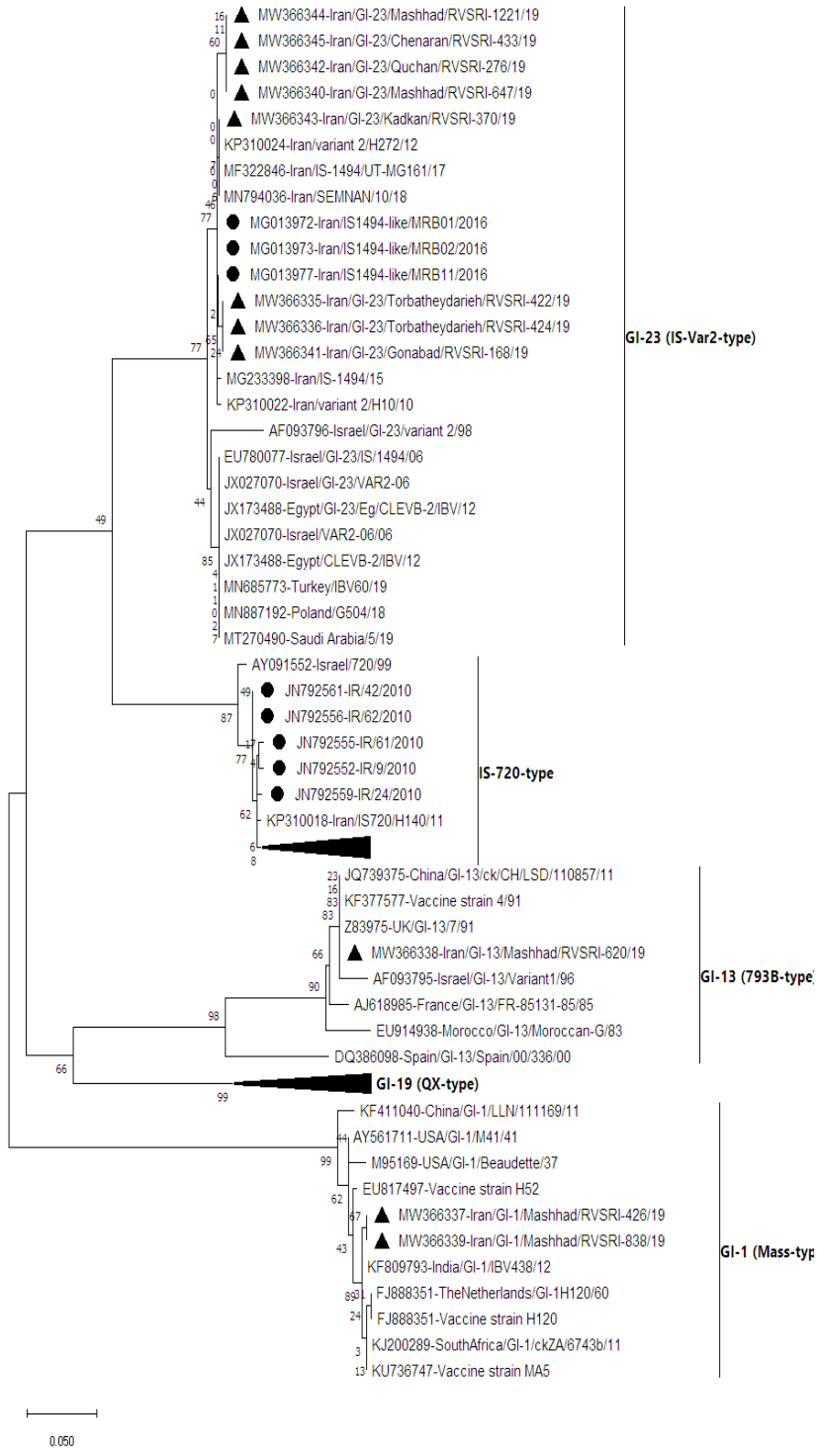

Figure 1.
Maximum Likelihood phylogenetic tree of the partial nucleotide sequences of the S1 gene.
The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model with 1000 bootstrap replicates. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 70 nucleotide sequences. There was a total of 314 positions in the final dataset. IBV strains previously identified in the Khorasan Razavi province are indicated by a filled circle. IBV strains identified in this study are indicated by a filled triangle. Evolutionary analyses were conducted in MEGA X.

This mutation that caused amino acid changes in the S1 protein, results in the formation of a distinct subbranch in the phylogenetic tree of protein sequences (Figure 3). It is noteworthy that this virus also had the chance to spread to adjacent cities (Figure 2).

In conclusion, this study shows that although the detected viruses originated in Iran, probably two IBVs with unreported point mutations have spread in two separate regions in the Khorasan Razavi province. Given that in one of these viruses detected in Torbat Heydarieh and Gonabad, a non-synonymous point mutation resulted in a change in the S1 protein, the virus may have different virulence and neutralizing epitopes than other GI-23 lineage viruses previously reported in Iran. Based on the information of this study, the possible prevalence of these viruses can be traced in future studies in Iran, and to improve biosecurity measures, possible routes of virus entry into different regions can be identified.

Materials & Methods

Sampling

15 commercial broiler and layer flocks with clinical signs and gross lesions suspected of IBV infection located in the Khorasan Razavi province were examined for IBV genotypes in 2019. The vaccination program in these flocks was mostly in the form of the Massachusetts vaccines and in some cases as a combination of Massachusetts and 793B vaccines. During necropsy, sampling of tracheal, renal, and cecal tonsil tissues was performed from 5 to 10 birds of each flock. After transferring tissue samples to the laboratory on the ice pack, the samples were stored at -70°C. Molecular tests were performed in the Research Department laboratory of Razi Vaccine and Serum Research Institute (RVSRI), Mashhad, Iran.

Viral RNA isolation

RNA isolation was performed using the High Pure Viral RNA Kit (Roche). The quality and quantity of the isolated genomic RNA were evaluated using NanoDrop 2000c spectrophotometer. Some of the isolated RNA was directly used to make cDNA, and the remaining RNA was stored at -70°C.

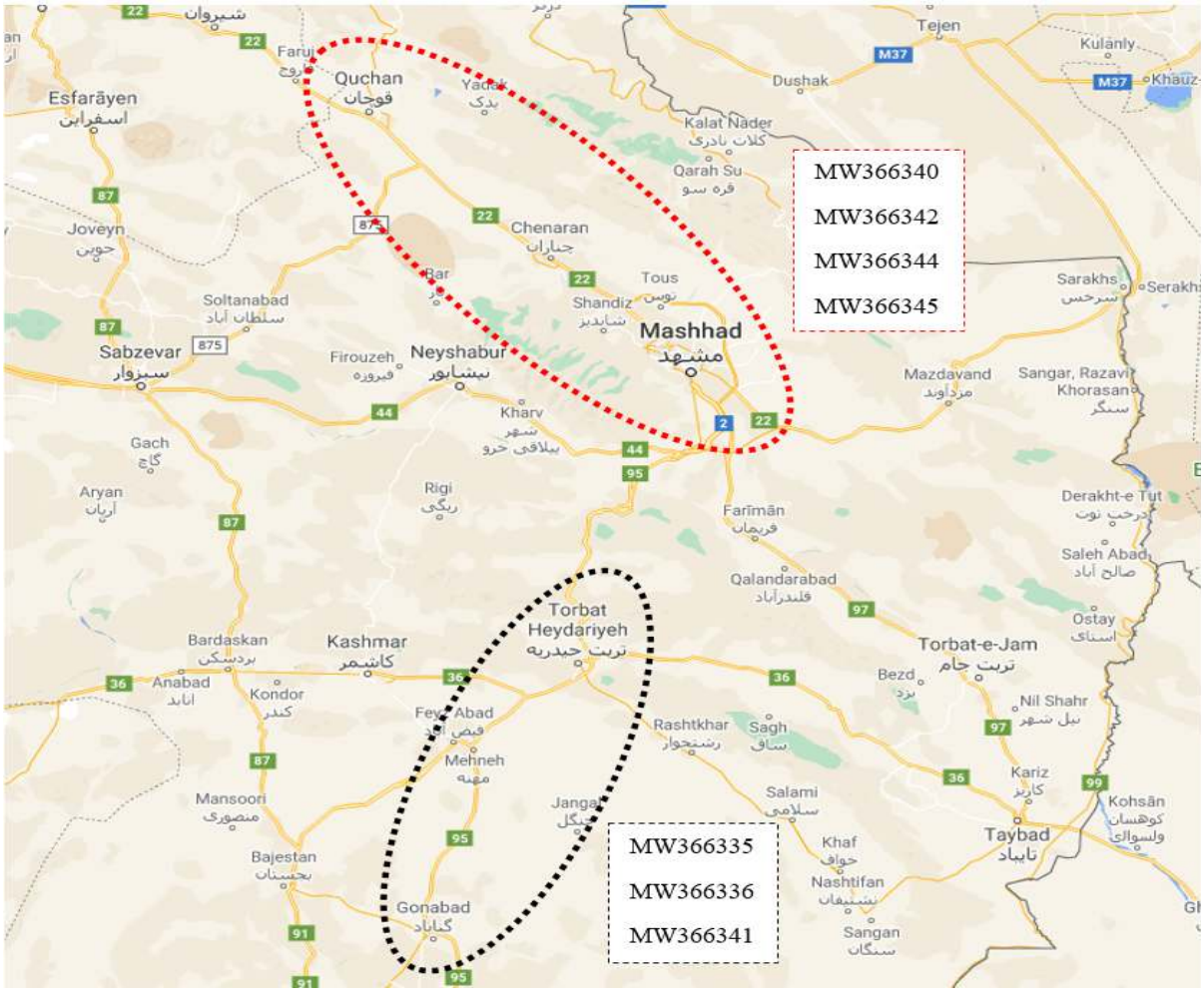

Figure 2.
Map of the prevalence of mutated IBV strains in two regions in the Khorasan Razavi province.
Viruses with 100% similarity have been identified in the areas shown inside an ellipse. The virus GenBank accession numbers are listed next to each ellipse. Google map image from: https://www.google.com/maps/@35.9244275,58.3266594,8z

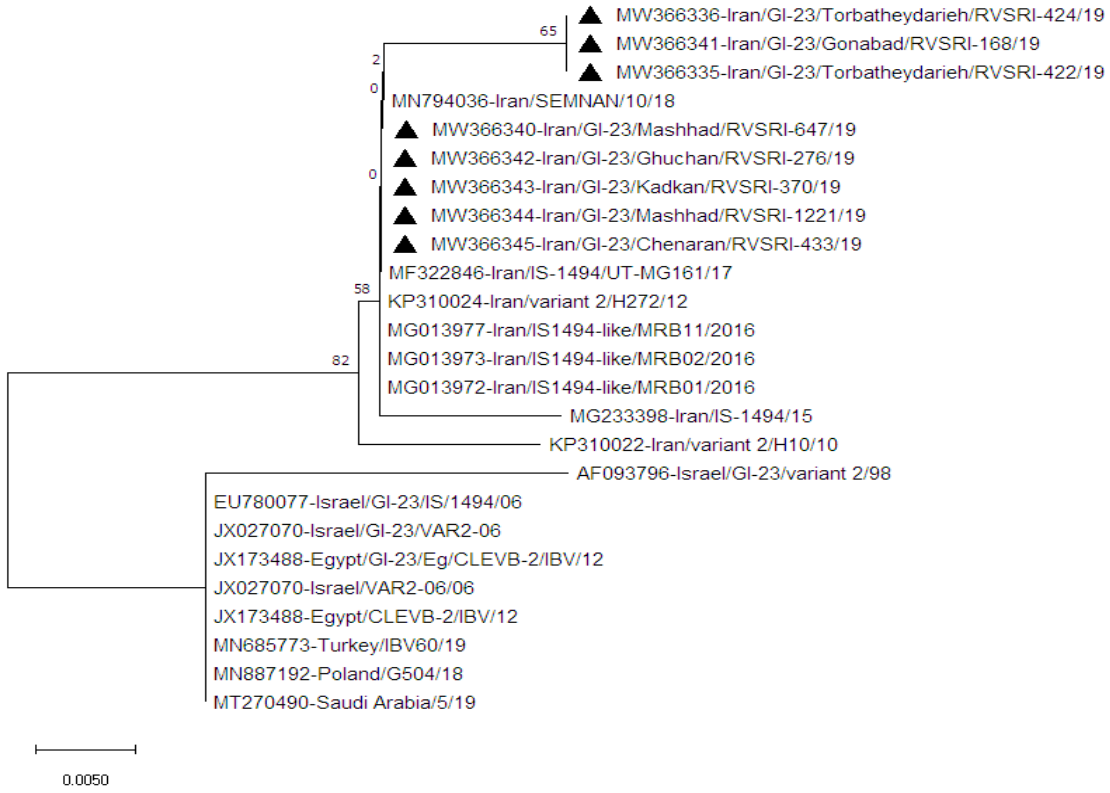

Figure 3.
Maximum Likelihood phylogenetic tree of the S1 partial gene amino acid sequences of GI-23 lineage viruses.
The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model with 1000 bootstrap replicates. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 25 amino acid sequences. There was a total of 107 positions in the final dataset. IBV strains identified in this study are indicated by a filled triangle. Evolutionary analyses were conducted in MEGA X.

Reverse Transcription (RT) Reaction

cDNA synthesis was performed using M-MuLV reverse transcriptase (RevertAid, Thermo Scientific, Carlsbad, CA, USA) immediately after RNA isolation. A specific primer of the S1 partial gene called SX2 was used to make cDNA (Table 1) [15]. The cDNA was employed for PCR and the remainder was stored at -20 °C.

PCR and nested PCR

PCR and nested PCR were performed using primers XCE1, SX2, SX3, and SX4 (Table 1) [15, 16]. These primers are common to most known strains of IBV and amplify a segment of the S1 gene that varies between IBV genotypes. PCR and nested PCR were performed in a total volume of 20 µl using a mixture of Taq polymerase 2X Master Mix (Ampliqon, Odense, Denmark), 1 µl (10 µM) of each forward and reverse primer, 1 µl of the template DNA, and 7 µl of sterile distilled water. Amplification was performed with a thermal profile (1 step of 95°C for 5 min, 35 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec, and 1 step of 72°C for 5 min) for PCR and nested PCR. For the first PCR, XCE1 and SX2 primers were used and the second PCR (nested PCR) was performed using the first PCR product (one microliter of one to ten dilution) as template and SX3 and SX4 primers. For all PCR steps, the negative control reaction consisted sterile distilled water instead of the template DNA. H120 and 793B vaccine viruses were used for positive control reactions to verify the performance of cDNA synthesis and PCR reactions.

Evaluation of nested PCR reactions and nucleotide sequencing

To evaluate the nested PCR for the presence of 393 bp band, gel electrophoresis was performed with 1.5% agarose. PCR products were sequenced (Bioneer, Korea) by using specific primers. The chromatograms obtained from the sequencing were examined and the results were edited as needed. Confirmed results were used for phylogenetic analysis.

Phylogenetic analysis of nucleotide and amino acid sequences

For phylogenetic analysis, the variable region sequences of the S1 gene related to 27 viruses with defined genotypes [7], were taken from GenBank (http://www.ncbi.nlm.nih.gov). By searching in GenBank, S1 gene sequences of 20 IBV strains related to the Khorasan Razavi province isolated from 2010 to 2016 were downloaded. Genetically similar sequences with strains detected in this study, based on BLAST results, included strains from Iran and other countries, were also obtained from GenBank for tree construction. Nucleotide alignment of these sequences was performed by ClustalW algorithm implemented in BioEdit software version 7.5.2 [17]. Phylogenetic analysis was performed using partial sequences of S1 genes in ME-

GA-X software [18]. After analyzing to find the best models for tree construction, the Maximum Likelihood statistical method, GTR + G substitution models, and test of phylogeny by bootstrap method with 1000 replications were used.

Due to the importance of studying amino acid changes in teh S1 protein, phylogenetic tree construction was performed based on partial S1 amino acid sequences of GI-23 lineage viruses. After analyzing to find the best models for tree construction, the Maximum Likelihood statistical method, JTT substitution model, and test of phylogeny by bootstrap method with 1000 replications were employed.

Table 1.
Sequence and position of the oligonucleotide primers used in PCR and nested PCR

Oligonucleotide	Sequence (5' - 3')	Position in S1 sequence	Reference
XCE1 +	CACTGGTAATTTTTCAGATGG	728 to 749	16
SX2 -	TCCACCTCTATAAACACCYTT	1148 to 1168	15
SX3 +	TAATACTGGYAATTTTTCAGA	705 to 725	15
SX4 -	AATACAGATTGCTTACAACCACC	1075 to 1097	15

Authors' Contributions

S-E.T., R.T. and H.F. conceived and planned the experiments. S-E.T., R.T., M.S., M.F. and E.V. carried out the experiments. S-E.T. carried out the nucleotide and phylogenetic analysis. R.T., N.MA., S.S., J.AA., M.G., M.T., M.F., N.K., M.KA. M.J.M., A.S., and T.M. contributed to sample preparation. S-E.T. contributed to the interpretation of the results. S-E.T. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research and analysis. All authors have read and approved the final draft of the manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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RESEARCH ARTICLE

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A newly discovered interference of the central nitrergic system on oxytocin-induced hypophagia in layer-type chickens

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ABSTRACT

Various neurochemical pathways are participating in the regulation of food intake in mammals and birds. Both oxytocin (OT) and nitric oxide (NO) are known as hypophagic agents in birds. This study consisted of 6 experiments and each experiment had 4 groups (ngroup=11, 5-day-old chickens). In all experiments, 3-hour food-deprived (FD3) birds received intracerebroventricular (ICV) injections either control diluent or drug solution. Then the birds had ad libitum access to the food and fresh water and then cumulative food intake (gr) was measured based on the percentage of the body weight (%BW). In experiments 1 to 3, ICV injections of L-arginine (precursor of NO, 200, 400, and 800 nmol), L-NAME (NOS inhibitor, 100, 200, and 400 nmol) and OT (2.5, 5, and 10 µg) were performed respectively. In experiment 4, each group received any ICV injections of L-NAME (100 nmol), OT (10 µg) or a co-injection of L-NAME (100 nmol) and OT (10 µg). In experiment 5, L-arginine (ICV, 200 nmol), OT (10 µg), or L-arginine (200 nmol) and OT (10 µg) were injected to the groups. Experiment 6 was similar to the experiment 5, although the dose of OT was 2.5 µg in all the treatment groups. Results showed that the ICV injection of L-NAME (100 nmol) significantly attenuated hypophagic effect induced by OT (10 µg) ($p < 0.05$). Findings suggested that NO might mediate the hypophagic effect of OT in FD3 neonatal layer-type chickens.

Keywords

L-NAME; L-arginine; food intake; bird

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Number of Tables: 2
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Number of Pages: 12

Abbreviations

%BW: percentage of the body weight
FD3: 3-hour food-deprived
ICV: intracerebroventricular
L-NAME: N(G)-Nitro-L-arginine methyl ester
NO: nitric oxide
NOS: nitric oxide synthase
OT: oxytocin
OXTR: oxytocin receptor

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Introduction

Various neurotransmitters play a role in the control of food intake via activation of different neurochemical pathways inside the central nervous system (CNS). Within the last few decades, physiologists have discovered many neurochemical pathways that control the feeding behaviors and have tried to find out the possible interactions among them [1–3]. Nitric oxide (NO) is a free radical gaseous molecule produced from L-arginine by the action of nitric oxide synthase (NOS). Neurons, glia and vascular cells can express NOS and produce NO inside the brain [4]. NOS-containing neurons located in the hypothalamus primarily are presented in the paraventricular nucleus of hypothalamus (PVN) and supraoptic nucleus (SON). Also, the axons of these neurons project to the pituitary gland [5]. NO has different physiological functions in the CNS including regulation of pain, memory, learning, neurotransmitter release, and feeding behavior in mammals and birds [3,6–8]. Previous studies have shown that the ICV injection of L-arginine (400 and 800 nmol) as a precursor of NO, significantly reduced food intake in neonatal chickens. On the other hand, ICV administration of NG-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, increased food intake in neonatal layer-type chicken [8,9]. Although, NO is known as a feeding-inhibitory molecule in layer-type chicken, but ICV injection of NO has feeding-stimulatory effect in mice [10] and broiler-type chicken [11]. These divergent reports may relate to genetic differences between mammals and birds or even the different genetic background in between the strains. Other studies have shown that the effects of NO on food intake are mediated by other neurotransmitters. For instance, ICV injection of L-NAME attenuated the anorexigenic effect of corticotropin-releasing hormone (CRH) in neonatal chickens, while Neuropeptide Y (NPY)-induced feeding behavior was not affected by L-NAME [12]. Furthermore, the mediatory role of NO on hypophagia induced by leptin has been reported in broilers and Leghorns [13].

Oxytocin (OT) is known as a nano-peptide neurotransmitter and is associated with parturition, lactation, cognition, tolerance, adaptation, and complex sexual and maternal behaviors [14]. Besides, OT has a role in the regulation of food intake in mammals and birds [15–17]. OXTR belongs to G protein-coupled group and primarily pairing with Gq proteins to phospholipase C [18]. OXTR is highly expressed in the regions that are involved in food intake regulation, such as the hypothalamus, nucleus accumbens, amygdala, ventral tegmental area, frontal cortex, insula, and hindbrain [19,20]. The OT-like neurohypophysial hormone has been identified in non-mammalian vertebrates such as birds and frogs called mesotocin (MT). Studies presented structural and functional similarities between mesotocin and mammalian oxytocin and showed that avian mesotocin receptor (MTR) is orthologous to mammalian oxytocin receptor (OXTR) [21]. Jonaidi et al. (2003) reported that OT plays a unique role in reducing feed intake by acting on mesotocin (MT) and/or vasotocin receptors in chickens [17]. The previously same stimulating activity of both MT/OT about ACTH release in the hypothalamic–pituitary–adrenal axis of birds has been confirmed [22]. Similar to the effect of OT in mammals [23], several investigations have shown the dose-dependent hypophagic effect of this neurotransmitter in birds [17,24].

The presence of the NOS in the magnocellular neurons in the brain suggests the mediatory role of NO in the production and release of OT. Also, several studies showed that ICV injection of NO donors significantly mediates OT production and release in laboratory animals [25,26]. For example, the recent research on rats showed that penile erection induced by OT significantly decreased after the injection of SMTC, an inhibitor of the neuronal NOS, into the bed nucleus of the stria terminalis (BNST). Based on this evidence, OT induces this physiologic behavior via the mediatory role of NO [27]. In another study, the analgesic effect of OT in mice was investigated and the mediatory action of NO was illustrated by the injection of L-arginine inside the spinal cord [28]. All of this evidence highlights the mediatory role of NO on OT-induced behaviors in the CNS. In addition, the anatomical relationship between NOS-containing neurons and oxytocinergic neurons [29] boosting the possible interaction between central nitrergic and oxytocinergic systems in birds. Nevertheless, no evidence has been reported so far about the interconnection of these two systems on food intake in chickens. To test this hypothesis, 6 experiments were performed on 3-hour food-deprived (FD3) neonatal layer chicken to find out the probable interaction of OT and NO on food intake behavior.

Results

The possible interaction between nitrergic and oxytocinergic systems on cumulative food intake in FD3 neonatal layer-type chickens was illustrated in "Fig. 1-6". In experiment 1, the ICV injection of 200 nmol L-arginine had no significant effect on cumulative food intake in comparison with the control group in 30, 60, and 120 minutes post-injection ($p \geq 0.05$). While the ICV administration of 400 and 800 nmol L-arginine significantly and dose-dependently decreased the food intake in comparison to the con-

trol group in all the time-points ($p < 0.05$). These results suggest the dose-dependent hypophagic effect of NO in neonatal layer-type chicken [Treatment effect: F (3, 40) = 1749.01, $p < 0.01$; time effect: F (2, 80) = 4813.53, $p < 0.01$; treatment × time interaction: F (6, 80) = 29.17; $p < 0.01$] (Fig. 1).

In experiment 2, the ICV injection of 100 nmol L-NAME made no significant changes in cumulative food intake in comparison with the control group in 30, 60, and 120 minutes post-injection ($p \geq 0.05$). However, ICV injection of 200 nmol and 400 nmol L-NAME significantly enhanced cumulative food intake in comparison to the control group in all the time-points ($p < 0.05$). This data shows the hypophagic effect of NO in neonatal layer-type chicken due to the administration of the NOS inhibitor (L-NAME) [Treatment effect: F (3, 40) = 2841.72, $p < 0.01$; time effect: F (2, 80) = 5825.12, $p < 0.01$; treatment × time interaction: F (6, 80) = 52.81; $p < 0.01$] (Fig. 2).

In experiment 3, the ICV injection of 2.5 µg Oxytocin did not significantly alter the cumulative food intake in comparison with the control group in 30, 60, and 120 minutes post-injection ($p \geq 0.05$). However, the ICV administration of higher doses of Oxytocin, 5 µg, and 10 µg, significantly decreased cumulative food intake in comparison with the control group in all the time-points ($p < 0.05$). These results illustrate the dose-dependent hypophagic effect of Oxytocin in neonatal layer-type chickens [Treatment effect: F (3, 40) = 1038.46, $p < 0.01$; time effect: F (2, 80) = 3274.82, $p < 0.01$; treatment × time interaction: F (6, 80) = 21.65; $p < 0.01$] (Fig. 3).

In experiment 4, ICV co-injection of 100 nmol L-NAME and 10 µg of oxytocin, significantly attenuated the hypophagic effect of oxytocin in 30, 60, and 120 minutes post-injection ($p < 0.05$). Despite, 100 nmol of L-NAME had no significant effect on cumulative food intake in comparison with the control group in all the time-points ($p \geq 0.05$). This data may reveal the mediatory effect of nitrergic system on the hypophagic effect of OT in neonatal layer-type chickens [Treatment effect: F (3, 40) = 3482.73, $p < 0.01$; time effect: F (2, 80) = 5037.12, $p < 0.01$; treatment × time interaction: F (6, 80) = 41.53; $p < 0.01$] (Fig. 4).

In experiment 5, ICV co-injection of 200 nmol L-arginine and 10 µg of oxytocin, significantly amplified the hypophagic effect of oxytocin in 30, 60, and 120 minutes post-injection ($p < 0.05$). However, the ICV injection of L-arginine (200 nmol) could not significantly change cumulative food intake in comparison with the control group in all the time points ($p \geq 0.05$). This data support the possible interaction between nitrergic and oxytocinergic systems on food intake and nitrergic system may have a synergistic effect on the regulation of food intake induced by oxytocin in layer-type chickens [Treatment effect: F (3, 40) = 2538.47, $p < 0.01$; time effect: F (2, 80) = 4281.06, $p < 0.01$; treatment × time interaction: F (6, 80) = 37.26; $p < 0.01$] (Fig. 5).

In the final experiment, experiment 6, neither ICV injection of 200 nmol L-arginine nor administration of 2.5 µg oxytocin could significantly alter the cumulative food intake in comparison with the control group in 30, 60, and 120 minutes post-injection ($p \geq 0.05$). While the co-administration of both drugs significantly decreased the cumulative food intake in comparison with the control group in all the time-points ($p < 0.05$). Based on these results, a synergistic collaboration between these two central systems on the regulation of food intake in layer-type chicken

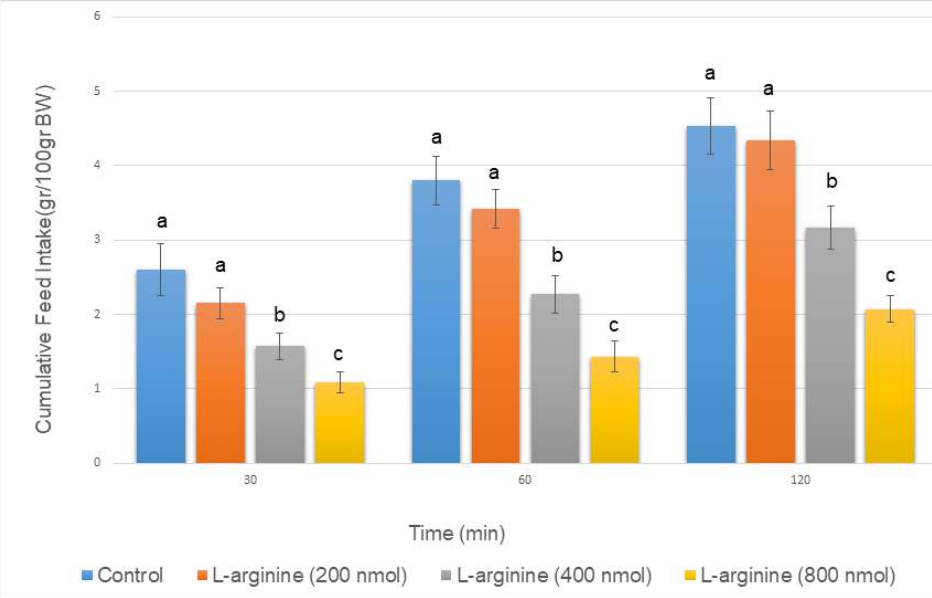

Figure 1.
Effect of ICV injection of L-arginine (200, 400 and 800 nmol) on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a, b and c; $p < 0.05$). L-arginine: precursor of NO

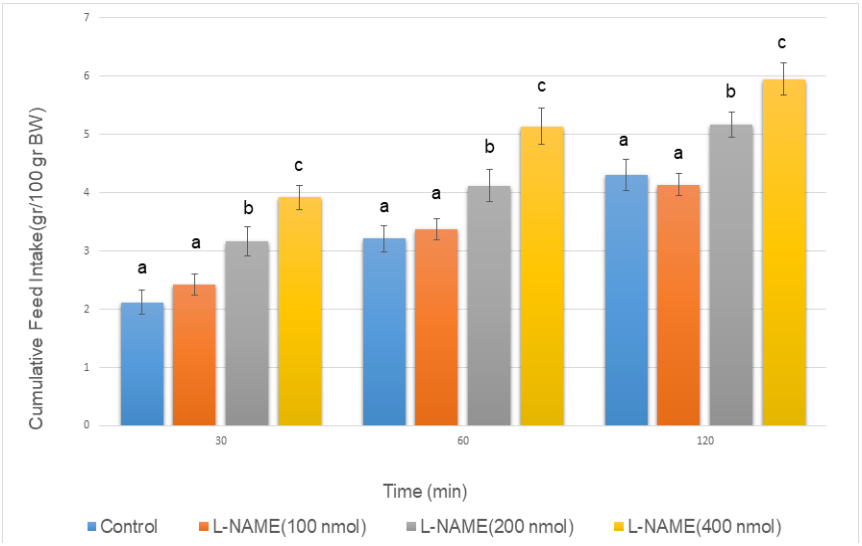

Figure 2.
Effect of ICV injection of L-NAME (100, 200 and 400 nmol) on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a, b and c; $p < 0.05$). L-NAME: NOS enzyme inhibitor

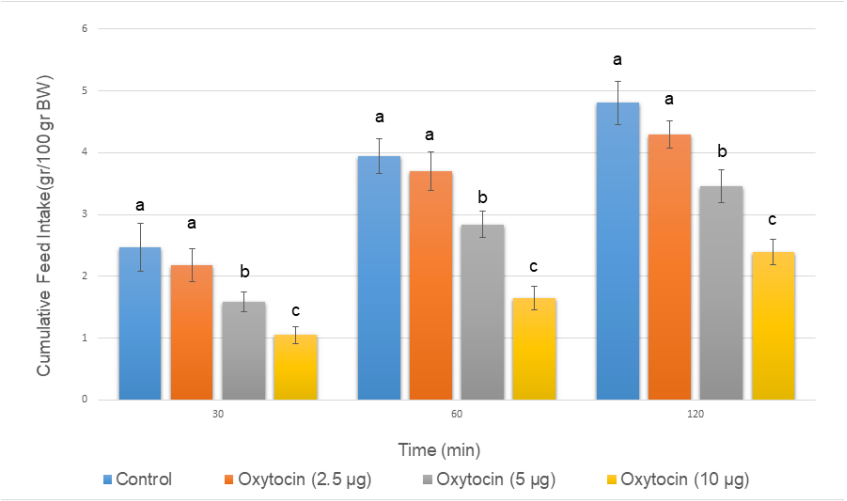

Figure 3.
Effect of ICV injection of oxytocin (2.5, 5 and 10 µg) on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a, b and c; $p < 0.05$)

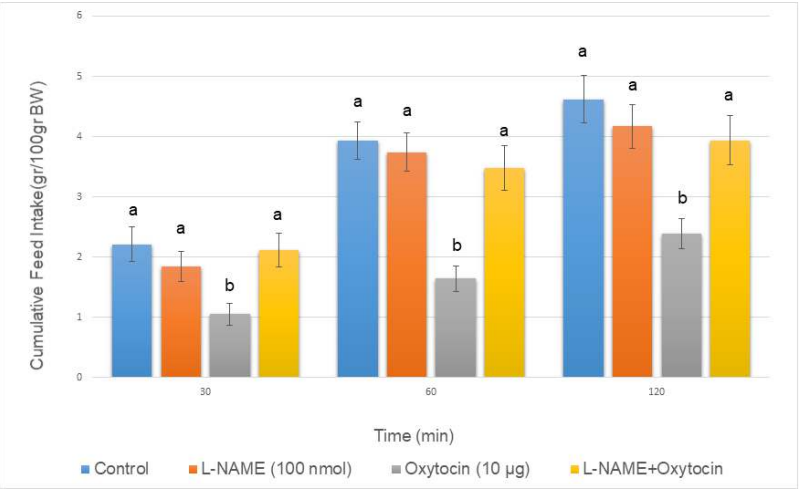

Figure 4.
Effect of ICV injection of L-NAME (100 nmol), oxytocin (10 µg) and their co-injection on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a and b; $p < 0.05$). L-NAME: NOS enzyme inhibitor

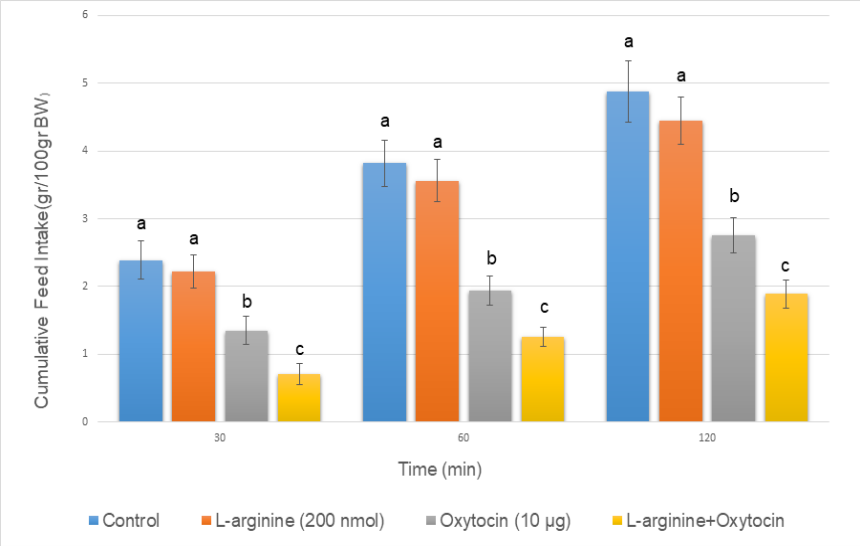

Figure 5.
Effect of ICV injection of L-arginine (200 nmol), oxytocin (10 µg) and their co-injection on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a, b and c; $p < 0.05$). L-arginine: NO precursor

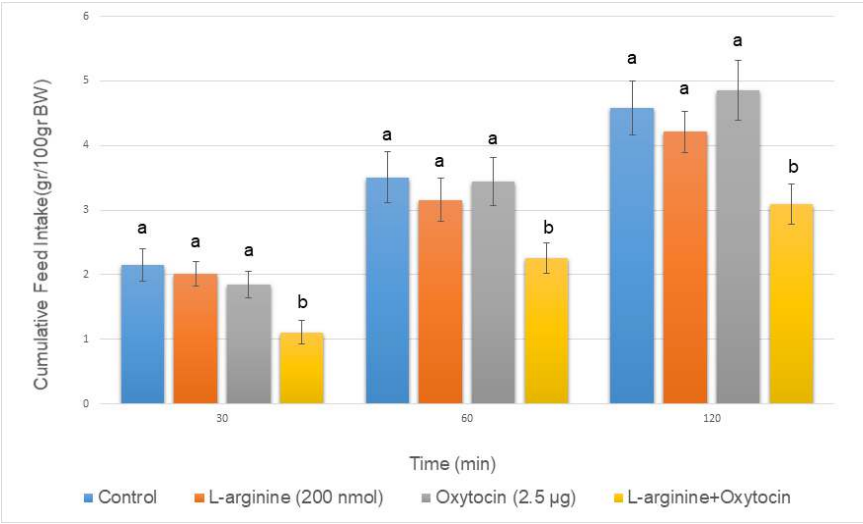

Figure 6.
Effect of ICV injection of L-arginine (200 nmol), oxytocin (2.5 µg) and their co-injection on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a and b; $p < 0.05$). L-arginine: NO precursor

is possible [Treatment effect: F (3, 40) = 1964.51, $p < 0.01$; time effect: F (2, 80) = 3275.83, $p < 0.01$; treatment × time interaction: F (6, 80) = 28.31; $p < 0.01$] (Fig. 6).

Discussion

This is the first report about the interaction between nitrergic and oxytocinergic systems on the regulation of food intake in FD3 neonatal layer-type chicken. Based on the results of this study, the ICV injection of L-arginine decreased the cumulative food intake in FD3 neonatal layers (Fig. 1). While the ICV administration of L-NAME increased the cumulative food intake (Fig. 2). Notably, some previous studies indicate an inconsistent finding regarding the effect of nitrergic system on food intake in layer-type and broiler-type chickens. For example, there is evidence that showed the intraperitoneal (IP) injection of L-NAME decreased feeding behavior in both layers and broilers [8], and the same has been recorded for IP injection of L-NAME in the rat [39]. The main reason for this discrepancy is probably due to the different routes of the administration, which could cause the involvement of peripheral NO receptors in the IP administration and consequently activation of different cascades on the food intake regulation. Choi et al. (1995) showed that the ICV injection of L-NAME had a hypophagic effect in broilers which is contrary to our findings [40]. On the other hand, the result of Khan et al. (2007) indicated that the ICV injection of L-NAME

increased food intake in layer-type chickens which is in agreement with our findings [8]. Another study revealed that the ICV injection of L-NG-Nitroarginine (L-NNA) a competitive NOS inhibitor, significantly diminished food intake in both broiler and Leghorn chicken which is in disagreement with our findings [13]. However, the other study about the interaction of nitrergic and cannabinoidergic systems showed the hypophagic effect of NO in neonatal layer-type chicken which is in agreement with the result of this study [38]. These opposite findings in layer-type and broiler-type chicken may be due to the different genetic characteristics between these strains. Additionally, different neurochemical pathways in feeding behavior are impressed by the different genetic backgrounds that eventually could cause even an opposite feeding response to the same neurotransmitters/neuromodulator [3,8,41–43].

OT is known as a regulator of food and water intake in mammals and OXTR(s) has been identified in the SON and PVN [15]. Also, this neurohormone is highly expressed in magnocellular neurons of the hypothalamus. It has been shown that both central and peripheral injections of OT have decreased food intake in mammals [18]. The hypophagic effect of OT in mammalian for the first time has been reported in the rat [15]. Kook et al. (1964) for the first time reported that the metabolic effects of OT in the chicken are the increased percentage of glucose and fatty acid in plasma and he showed that the responsive receptors to OT are expressed in the CNS of the bird those later has been discovered and called MTR by scientists as OXTR homologous [44]. Besides for the first time, Jonaidi et al. (2003) showed that ICV injection of OT could dose-dependently decreased feeding in meat-type chickens [17]. Furthermore, Mirnaghizadeh et al. (2017) confirmed the hypophagic effect of OT in neonatal broiler-type chickens [24]. These studies are in accordance with our results which presented the hypophagic effect of OT instead of MT on food intake in FD3 neonatal layer-type chicken again.

Several suggestions for the underlying mechanism of OT-induced hypophagia in mammals have been proposed. For instance, after ICV injection of OT, PVN neurons modulate intrinsic brainstem reflexes and directly control the vagal efferent projections toward the gastrointestinal system to inhibit gastric motility and ingestion [18]. Another suggestion is again related to PVN neurons, especially OT projections associated with melanocortin-4 receptors (Mc4R s) and they are a key point in food intake regulation [45,46]. The inhibitory effect of hypothalamic pro-opiomelanocortin (POMC) neurons and melanocortin-3/4 receptors on food intake have also been demonstrated in birds [47,48]. Nevertheless, the exact underlying mechanism of OT on food intake regulation in chicken has not been elucidated yet, however, OT or MT in birds may regulate food intake via similar pathways as in mammals.

Based on the literature we suppose the mediatory role of NO about food intake-induced by OT in chickens. The results of our study showed that NO has a mediatory effect on the hypophagic effect of OT in layer-type chickens and it has been reported that following the injection of NO donors and L-NAME into the lateral ventricle of the brain, release of the other neurotransmitters are altered in the different nuclei of the hypothalamus, especially in magnocellular neurons, which are involved in OT secretion. The presence of the NOS in the circumventricular organs and magnocellular neurons supports our results about the modulatory effect of NO on OT [49]. It is also suggested that NO increases the outputs of PVN and SON that can increase OT secretion in magnocellular neurons and ultimately modulate OT-physiological behaviors. This could be a possible explanation for the mediatory role of NO on the hypophagic effect of OT and that seems it is a synergistic one.

PVH also contains PVH containing nitric oxide synthase-1 (Nos1PVH) which is projected to the spinal cord and hindbrain and is involved in feeding behavior. It might be considered that OT-induced hypophagia is modulated by signaling of the Nos1PVH neurons in the brain. Also, several neuropharmacological studies indicated that PVH contains Sim1-expressing cell type (Sim1PVH) which is playing an important role in the control of food intake. In addition, Nos1PVH neurons are a subset of Sim1PVH neurons and oxytocin-expressing PVH neurons (OXTPVH) known as a subset of Nos1PVH neurons [50–52]. This evidence can be another clue to support our finding regarding the possible interaction between nitrergic and oxytocinergic systems in control of food intake in birds such as what has been already identified in mammalian.

Besides, the mediatory role of NO has been reported in negative inotropic and chronotropic effects induced by OT in the heart, and blockage of the NOS by systemic administration of L-NAME could decrease the protective effect of OT on myocardial cell [53,54]. Gutkowska and Jankowski (2009) mentioned that the cardioprotection effect of OT is dependent on the activation of intrinsic cardiac cholinergic neurons and NO release is the major underlying mechanism [55]. This finding indicated the mediatory role of NO on OT outside of the CNS. On the contrary, Reis et al. (2007) illustrated that endogenous NO could act as an inhibitory effect on OT secretion to keep fluid homeostasis of the body in mammals [25]. This inconsistency might be due to OT release following the

ICV injection of angiotensin II and turning on another neural pathway, which is before NO, and this could alter the modulatory response of NO on OT release. Another study regarding the interaction of nitrergic and oxytocinergic systems outside of the CNS showed that the protective effect of OT in renal and hepatic injury induced by renal ischemia/perfusion in the adult male albino rat is probably NO-dependent. In this study administration of L-NAME before OT partially reversed the protective effect of OT and it seems that the protective effect of OT is dependent on NO production [56].

OT stimulates NOS and increases NO release in the CNS [5]. Also, ICV administration of OT enhanced NO and dopamine production via activation of neural pathways in the PVN and eventually related behavioral responses [27]. Canteros et al. (1995) suggested that increasing intracellular ionized Calcium and formation of Ca2+-calmodulin complex is the mechanism underlying activation of OT neurons in the hypothalamus and later this could activate the neural nitrergic system [57]. So far, the result of our study and other evidence are suggesting that oxytocin regulates NO release by stimulating NOS activity in the hypothalamus, while the oxytocinergic system is modulated by the nitrergic system. To support this finding, Nomura et al. (2005) showed the involvement of neuronal NOS-derived in the regulation of oxytocin gene expression in the hypothalamus [58]. Also, the up-regulation of NOS-mRNA by oxytocinergic neurons was demonstrated in the hypothalamo-neurohypophysial system [59]. These studies are supporting our findings and plotting a possible mechanism for the interaction of these two systems, although further investigations to elucidate the exact underlying signaling mechanism in birds are still needed.

Furthermore, some studies have shown that sexual and hormonal functions of OT in the hypothalamus such as regulation of the preovulatory gonadotropin-releasing hormone (GnRH) surge is a NO-dependent mechanism and can be amplified by injection of NO donors [60,61]. These results are in agreement with the results of our study and this could be considered as another clue to support our hypothesis about the possible interaction between these two systems.

We have observed functional synergistic interaction between nitrergic and oxytocinergic systems on the regulation of food intake in FD3 neonatal layer-type chickens and we found that OT-induced hypophagia is NO-dependent in the neonatal layers.

Materials & Methods

Animals

In order to investigate the possible interaction between NO and OT in the regulation of food intake, female one-day-old layer-type birds were bought from a local hatchery (Morghak Company, located in Tehran, Iran, n=264) and they were kept together for two days. After this, the chickens were moved into the individual cages randomly. Cage temperature and relative humidity were maintained at 32 C ± 1 (Electrical heat) and 40–50 % respectively. The lighting/dark phase was 23:1 hr [30,31]. All birds throughout this study had free access to a commercial starter diet and freshwater. It was consisting of 21% crude protein and 2850 kcal/kg metabolizable energy (Animal Science Research Institute Co. Iran, Table 1). The chicken was deprived of food for three hours (FD3) before any ICV injection, however, freshwater was available all the time. The initiation of the ICV injection was when the chickens were five-day-old. All the procedures were performed according to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health, USA (publication No. 85-23, revised 1996) and were approved by the Institutional Animal Ethics Committee of Faculty of Veterinary Medicine, University of Tehran.

Experimental drugs

All of the experimental drugs including L-arginine (precursor of NO), Oxytocin (OT), N(G)-Nitro-L-arginine methyl ester (L-NAME, nitric oxide synthesis inhibitor) and Evans blue were bought from Sigma Co. (Sigma, USA). All drugs were dissolved in absolute dimethyl sulfoxide (DMSO) initially and later were diluted with 0.85 % saline containing Evans blue at a ratio of 1:250.

ICV injection procedures

Six experiments were designed and each one had four groups (A–D) (ngroup=11). Chickens were assigned into the groups based on their body weights (scale GF-6100, Japan accuracy = 0.01g). Therefore, maximum consistency was achieved based on the bodyweight in the groups. In every experiment, the non-anesthetized birds were injected intracerebroventricularly by a microsyringe (Hamilton, Switzerland) only once [32,33]. In this technique, the bird's head was supported with an acrylic device in which the bill holder of the device made a 45° angle with the table and the scalp was parallel to the tabletop [34]. A plate with a fenestra was immediately located above the skull over the right lateral ventricle. A microsyringe was inserted into the right ventricle via the fenestra and the tip of the needle pierced just 4 mm below the skull skin and the solution was discharged through the ventricular fluid gradually (volume of each injection was 10µL). Notably, this route of injection causes no physiologic stress in the neonatal birds [35]. Evans blue was added into all injections and at the end of the experiment, chicken was decapitated immediately and by observing the blue color inside the ventricles the corrected ICV injection was confirmed. Data for statistical analysis was only obtained from those individuals who had the correct injection (n correct injection = 9-11) [36,37].

Food intake measurement protocol

In experiment 1, chickens in the group (A) received ICV injection of control solution and in the group (B), (C) and (D), they were administered by 200, 400, and 800 nmol of L-arginine respectively. Experiments 2 and 3 were conducted similarly to experiment 1, however, in experiment 2, the different doses of L-NAME 100, 200, and 400 nmol and experiment 3, the different

doses of OT 2.5, 5, and 10 µg were injected into the lateral ventricle respectively. In experiment 4, chickens in the group (A) were received ICV injection of control solution, group (B) was injected with L-NAME (100 nmol), group (C) was administered with Oxytocin (10 µg) and group (D) received a co-injection of L-NAME (100 nmol) and oxytocin (10 µg). Experiment 5 was conducted similarly to experiment 4, except L-arginine (200 nmol) was used instead of L-NAME (100 nmol) in group (B) and group (D) received the co-injection of L-arginine (200 nmol) and oxytocin (10 µg). Experiment 6 was conducted similarly to experiment 5 and only a different dose of oxytocin (2.5 µg) was administered in groups (C) and (D) (Table 2). Immediately after the injection, chickens were returned to their individual cages and ad libitum food (pre-weighed) and water were provided. Then, the cumulative food intake was recorded at 30, 60, and 120 minutes post-injection by reweighting of the food. In order to decrease the effect of the bodyweight on the food intake volume, the food intake calculation was based on the bodyweight percentage (% BW). Following Jonaidi et al. (2003) study and based on our latest published articles about hypophagic role of OT in chicken [24,38], we have used OT instead of MT and the suggested doses to stimulate related receptors inside the brain.

Statistical analyses

Cumulative food intake was analyzed by repeated measure two-way analysis of variance (ANOVA) and is presented as the mean ± SEM. For treatments found to affect according to the ANOVA, mean values were compared with the Bonferroni test. p values < 0.05 were considered to indicate significant differences between treatment groups. The analysis of variance was performed using the model as given: $Yijk= \mu + \alpha j + \beta k + (\alpha\beta)jk + \varepsilon ijk$ Where Yijk is the value of its individual observation for valuables, µ: the grand mean, αj: is the treatment effect for the time, βk: is the treatment effect for the drugs, (αβ)jk: is the interaction effect for the time and drugs, εijk: error.

Table 1.
Ingredient and nutrient analysis of the experimental diet

Ingredient	(g/kg)	Nutrient analysis	(g/kg)
Maize	528.5	ME, MJ/kg	11.9
Soybean meal, 48% CP	315.7	Crude protein	210
wheat	50	Linoleic acid	17
Gluten meal, 61% CP	25.0	Crude fiber	36
Wheat bran	24.7	Calcium	10
Di-calcium phosphate	19.2	Available phosphorus	5
Oyster shell	12.3	Sodium	1.5
Soybean oil	10.0	Potassium	9.6
Mineral premix	2.5	Chlorine	1.7
Vitamin Premix	2.5	Choline	13012
Sodium bicarbonate	2.1	Arginine	11.4
Sodium chloride	2.0	Isoleucine	7.3
Acidifier	1.5	Lysine	12.1
DL-Methionine	1.0	Methionine	4.9
Toxin binder	1.0	Methionine + Cystine	8.3
L-Lysine Hcl	0.5	Threonine	7.0
cholecalciferol	1.0	Tryptophan	2.0
Multi enzyme	0.5	Valine	7.8

ME: metabolizable energy, CP: crude protein, per kg of diet, the mineral supplement contains 35.2 g manganese from $MnSO_4.H_2O$; 22g iron from $FeSO_4H_2O$; 35.2 g zinc from ZnO; 4.4 g copper from $CuSO_4.5H_2O$; 0.68 g iodine from ethylenediamine dihydroiodide; 0.12 g selenium from Na_2SeO_3. The vitamin supplement contains 1.188 g of retinyl acetate, 0.033 g of DL-α-tocopherol, 1.32 g of menadione, 0.88 g of thiamine, 2.64 g of riboflavin, 13.2 g of nicotinic acid, 4.4 g of pantothenic acid, 1.76 g of pyridoxine, 0.022 g of biotin, 0.36 g of folic acid and 1500 mg of choline chloride.

Table 2.
Intracerebroventricular injections in experiments

group	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
A	CS*	CS	CS	CS	CS	CS
B	L-arginine[¡]	L-NAME[¥]	OT[$]	L-NAME	L-arginine	L-arginine
	(200 nmol)	(100 nmol)	(2.5 µg)	(100 nmol)	(200 nmol)	(200 nmol)
C	L-arginine	L-NAME	OT	OT	OT	OT
	(400 nmol)	(200 nmol)	(5 µg)	(10 µg)	(10 µg)	(2.5 µg)
D	L-arginine	L-NAME	OT	L-NAME+OT	L-arginine+OT	L-arginine+OT
	(800 nmol)	(400 nmol)	(10 µg)	(100 nmol)+(10 µg)	(200 nmol)+(10 µg)	(200 nmol)+(5 µg)

*CS: Control solution
[¥]L-arginine: NO precursor
[¡]L-NAME: NG-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor
[$]OT: Oxytocin

Authors' Contributions

M.Z. and M.K. conceived and planned the experiments. H.Z. and B.R. carried out the experiments. A.B., M.Z. and K.M. contributed to the interpretation of the results. M.K took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Acknowledgments

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Competing Interests

The authors declare that there is no conflict of interest.

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Common Carp (*Cyprinus carpio*) parasites diversity and prevalence in Erbil aquacultures: gills, skin and intestinal infections

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ABSTRACT

This study was carried out to investigate the causative agents of the parasitic diseases affecting common carp (*Cyprinus carpio*) in aquacultures in the Erbil region. At first, all fish were visually examined, then, microscopic analysis of mucus, skin and fins scrapings and gastrointestinal contents were carried out, confirming that carps are infested by a large diversity of parasites, predominantly affected by *Dactylogyrus sp.*, followed by *Trichodina sp.*, and copepod parasites, with an infection rate of 25.2%, 17.2%, and 13.2%, respectively. On the other hand, the highest mortality was due to infestation by *Trichodina sp., Dactylogyrus sp., Ichthyophthirius sp.* and *Gyrodactylus sp.*, rating 40%, 35%, 29% and 28%, respectively. Several injuries and ulcerations were observed within gills, over fins and skin of infected fish. In conclusion, the results showed that carp fish from Erbil city are infested by several parasites causing pathological and mechanical injuries, which were associated with high mortality rates in carps.

Keywords

diversity, prevalence, carp, parasites

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Abbreviations

CC: common carp
C. carpio: Cyprinus carpio
PP: Potassium permanganate

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Introduction

Fish is an important food source for the world. Indeed, it is the human's single most important resource of high-quality protein, providing around 16% of animal protein [1,2]. Among numerous species of fishes, carps belong to the *Cyprinidae* family which is the biggest family of freshwater fishes, spreading widely around the world [3]. In Iraq, this fish is considered one of the most prized sources of protein. Due to their nutritive value, huge numbers of people, all over the country are developing carp aquacultures as a means of livelihood and/or income [4]. These observations suggest that a consistent source of fish is essential for the nutritional and financial health of a large segment of the worldwide population.

However, several disease agents (virus, bacteria and parasites) infect fishes [5], and a majority of freshwater fishes carry heavy parasitic infections, which deteriorate their food value [6]. The impact of parasites on fish health can be mechanical, or affecting the physiology and reproduction, or may even result in their death [7]. Various parasitic infections have been reported in the common carp (CC; *C. carpio*) [8, 9, 10]. Additionally, different types of parasitic infections have been recorded in this species in Iraq [8, 11, 12]. This parasitic fauna includes E*uglenozoan, Microsporidian, Ciliophorans, Myxozoans, Trematodes, Monogeneans, Cestodes, Nematodes, Acanthocephalans, Annelids, Molluscan,* and *Arthropods* [8, 11].

In addition, several areas of Iraq (Salah Al-Deen province, Babylon, Al-Diwaniyah, Kurdistan, and Najaf al-Ashraf) have been affected by carp infections due to parasitic agents [5, 8, 11, 1, 12]. Knowing that parasites can have harmful effects on carp populations or play a critical role in their mortality or growth retardation, leading to economic losses for the aquacultures, it has become important to carry out health monitoring on farmed fish in order to improve aquaculture systems and the food value of these fishes. Research on fish parasites and parasitic diseases that may reduce their growth and survival is imperative; therefore, the aim of this study was to find the parasitic disease agents that affect aquacultures of CC fish in Erbil city.

Results

Parasite diversity and prevalence

After examining 250 individual fish, several parasites were identified (Table 1). We identified *Ichthyophthirius sp., Trichodina sp., Dactylogyrus sp., Gyrodactylus sp., Bothriocephalus sp., Capillaria sp., crustaceans* (copepods), and leeches (Figures 1 and 2). Moreover, we observed other organisms on the gill—*Rotifera, Chlorophyta, Nematodes* and eggs of copepods. (Figure 2, C–G). This was the first identification of *Rotifera* (*Brachionus sp.*) in the gills of fish in Iraq.

Among all the identified parasites, the most predominant was *Dactylogyrus sp.*, with an overall prevalence of 25.2%, followed by *Trichodina sp.* and copepod parasites, with their global infection rates at 17.2% and 13.2%, respectively (Table 1). In the other taxa, the global prevalence is situated between 2% and 6% (Table 1). Surprisingly, the presence of *Capillaria sp.* infecting the gills was confirmed.

Four parasites (*Trichodina sp., Dactylogyrus sp., Ichthyophthirius sp.* and *Gyrodactylus sp.*) seemed to be associated with the highest mortality rates among the infected fish, 40%, 35%, 29% and 28%, respectively (Table 1). The remaining parasites were found in a small number of dead fish, with rates ranging between 10% and 18% (Table 1).

Clinical signs

Results of the analysis of the different infected fishes revealed the presence of several clinical signs (Table 2). Indeed, we observed the appearance of necrotic areas, ulcerations and hemorrhage on the gills. On others body parts, we found *Protozoa and Monogeneas* parasites. We also observed carps infected with crustaceans, tapeworms, nematodes and leeches that caused inflammations and mechanical injuries (see Figure 3).

Treatment results

Formalin and potassium permanganate (PP) were effective treatments against a majority of the identified parasites reported in this study.

Discussion

Fishes are recognized as an excellent food source for humans and are preferred as the perfect diet because of the higher proportions of unsaturated fatty acids [17]. Thus, this makes fish the source of basic income for millions of people worldwide [2]. However, in the last few years, several parasitic species have been reported in fishes [9], especially in CC [1, 5]. Indeed, several studies around the globe have revealed cases of infection in carps with many parasites [5, 7, 10]. These parasitic infections can cause great mortality rates and morbidity among them, which may disrupt personal or national wealth systems [18].

In the present study, laboratory analyses were performed on a set of 250 fish collected from five farms rearing CC in Iraq. All the parasitic species found have been previously identified in CC [9]. Thus, we observed eight parasite taxa: *Ichthyophthirius sp., Trichodina sp., Dactylogyrus sp., Gyrodactylus sp., copepods, Bothriocephalus sp., Capillaria sp.* and leeches.

Table 1.
Frequency of isolated parasites

Group	Species	No. of infected fish (%)	Habitat	Frequency of infection in examined aquacultures	No. of fish deaths (%)
Protozoa	*Ichthyophthirius sp.*	28 (5.6)	Gill, skin	3–5	8 (29)
	Trichodina sp.	86 (17.2)	Gill, skin, fins	10–20	35 (40)
Monogenea	*Dactylogyrus sp.*	130 (25.2)	Gill	12–30	46 (35)
	Gyrodactylus sp.	32 (6.4)	Gill, skin	3–5	9 (28)
Crustacean	*Copepods*	66 (13.2)	Gill, skin, fins	9–14	7 (11)
Cestodes	*Bothriocephalus sp.*	22 (4.4)	Intestine	3–4	4 (18)
Nematodes	*Capillaria sp.*	18 (3.9)	Intestine, gills	3–5	3 (17)
Annelida	*Leeches*	10 (2)	Gills	2–3	1 (10)

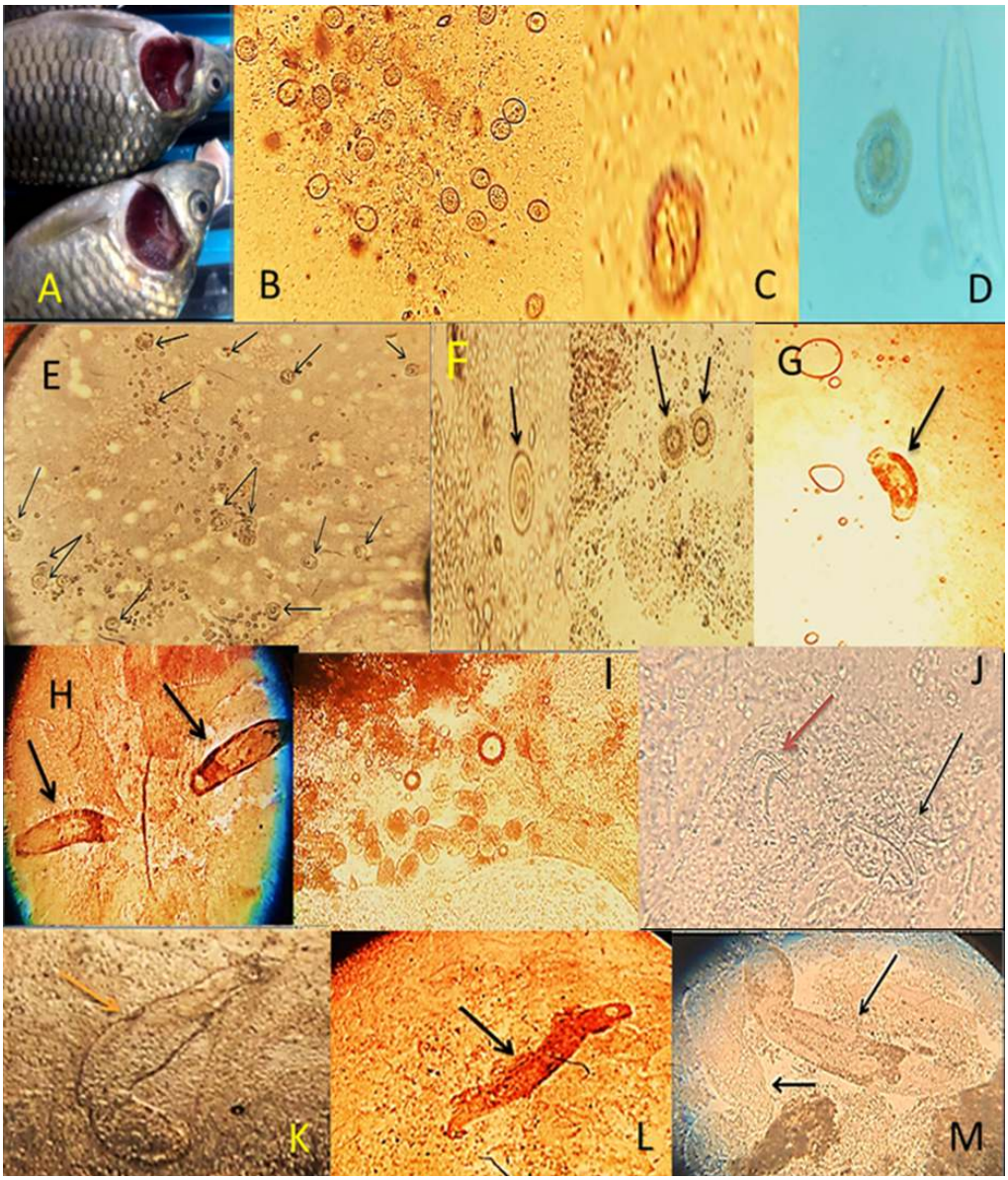

Figure 1
Detected Protozoa and Monogeneas parasites. A) Infected fish with *Ichthyophthirius*, B-C) *Ichthyophthirius sp.*, 400X and 1000X, E-F) *Trichodina sp.* shown by arrows, 400X, 1000X, G-I) *Dactylogyrus sp.*, arrows refer to the worm, 400X, J) *Dactylogyrus sp.*, black arrow refers to the haptor and red arrow to the hooks, 1000X), K-M) *Gyrodactylus sp.*, arrows refer to the worm, 400X.

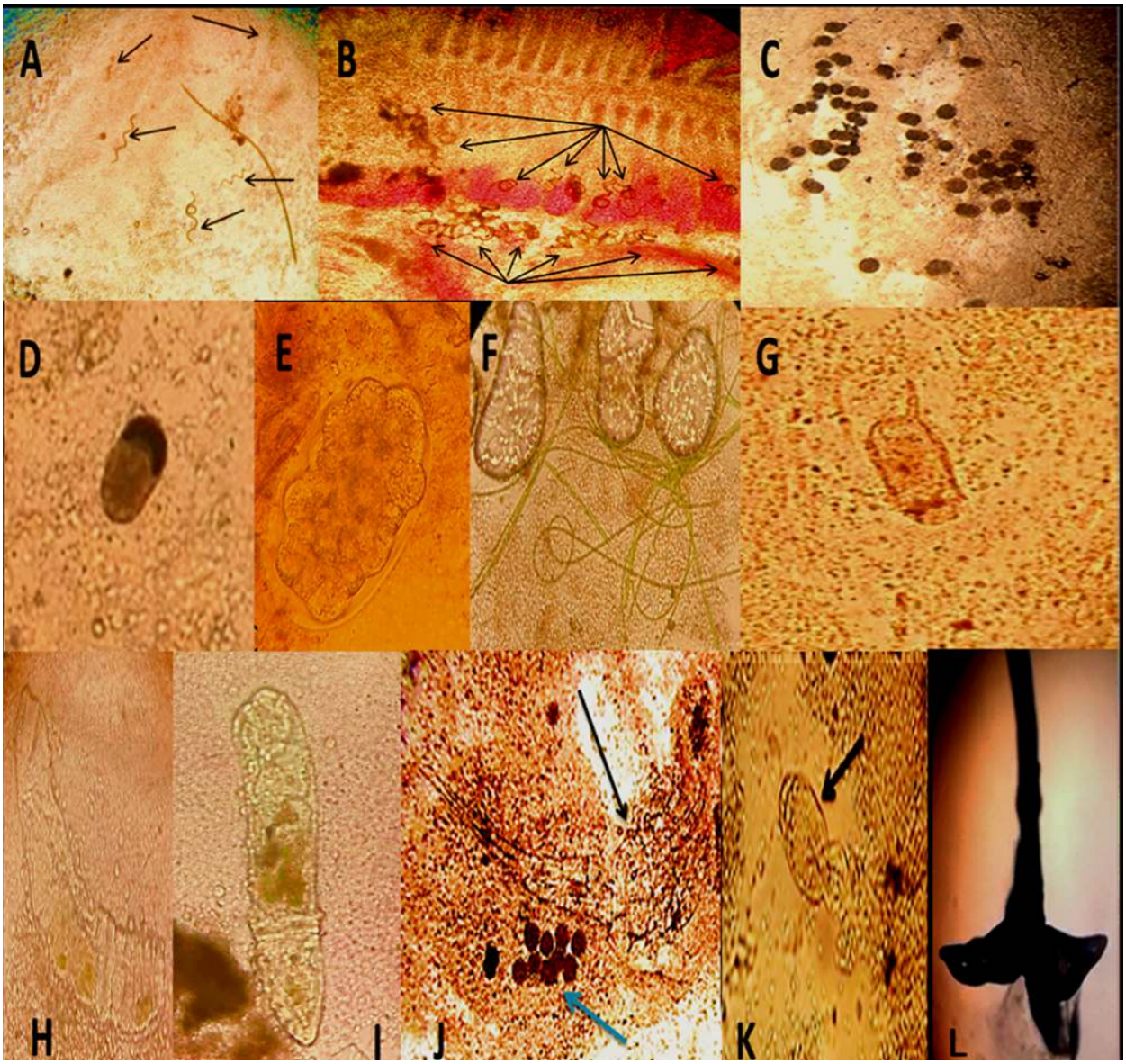

Figure 2
Detected Nemadodae, Crustacean and other organisms on fish gills. A-B) Nemadodae, capillaria worms from gill, arrows refer to the worm, 400X, C-E) Egg types detected on gills, C: Copypod, D and E: Nemadodae, 400X, F) *Chlorophyta* (green algae), G) R*otifera Brachionus sp.*, detected on gills, 400X, H-I) Leeches isolated from gills, 40X, J-L) Crustacean samples detected on gills and skin, black arrows refer to the crustacean, the blue arrow refers to the eggs. 40X, L) *Lernaea* anchor.

The most prevalent were protozoa and Monogeans (Table 1).

Regarding the protozoa group, two genera were found in the carps involved in our study: I*chthyophthirius sp.* and *Trichodina sp.* These parasites have been previously reported in Iraq [12, 19]. Among both protozoa, the most represented was *Trichodina sp.*, with 17.2% of carps infected with this parasite. This was found in the gills, skins, and fins. This observation is consistent with the results of previous studies. Indeed, Al-Marjan and Abdullah, 2009 found this parasite in the skin, fins, and gills of *C. carpio* from the Ainkawa fish hatchery in Erbil province [19]. The infection level detected in our study was less compared to previous studies [20]. Concerning the *Trichodina sp.*, we think that this identified species could most likely be *Trichodina reticulata* or *Trichodina nobilis*, which have been observed in carps from Iraq [20, 4]. However, the *Ichthyophthirius* genus parasites were found in the gills and skins, with a prevalence of 29%. This parasite is recognized by colonizing areas known in this geographical region, and has been reported in several areas of Iraq, infecting many fish species including *C. carpio* [12, 7, 19]. The second ciliated parasite belonging to the *Ichthyophthirius* genus could be the *Ichthyophthirius multifiliis* species.

Concerning *Monogenea*, two parasites were identified as *Gyrodactylus sp.* and *Dactylogyrus sp.* Our results showed that the most common parasite was *Dactylogyrus sp.*, with a rate of 25.5% within the

Table 2.
Common signs noted in infected fish

Type of Parasites	Common signs noted in infected fishes	Type of Treatment	Effectivity of the Treatment
Ichthyophthirius sp.	Lesions and necrotic area, white spots, paleness of gills	Formalin, potassium permanganate	100%, 95%
Trichodina sp.	Irritant area, gill filament fusion, congestion, ulceration and lesions on skin and gills, gill whitening	Formalin	100% repeated three times
Dactylogyrus sp.	Extreme amount of mucous, hemorrhage, gill necrosis, gill whitening and congestion	Salt baths, formalin	80%, 95%
Gyrodactylus sp.	Hemorrhage, ulceration, body inflammation, mechanical injury	Common salt 3%, potassium permanganate	70% for 3 days, 80%
Crustacean	Ulceration, inflammation, mechanical injury	Fenbendazole	80% for 3 days
Cestodes, Nematodes	Inflammation, redness, paleness, gill mechanical damage, congestion	Malathion	100%
Leeches	Inflammation, redness, paleness, gill mechanical damage, congestion	Malathion	100%

Monogean and other identified parasite taxa in this study. This species is more prevalent than the *Gyrodactylus sp.* (6.4%). The *Dactylogyrus* genus has been known as a parasite of various freshwater fishes, although most commonly found in the *C. carpio* in Iraq [1, 18]. However, many species of these two parasitic genus of *Gyrodactylus* and *Dactylogyrus* are known to infect many fish species [4]. Observation of both these monogenic parasites in farms can be seriously problematic, due to fact that their presence has been associated with the death of fish in carp farms [17]. This observation could explain the observed high death rates of 35% and 28% (Table 1), respectively, for *Dactylogyrus sp.* and *Gyrodactylus sp. Dactylogyrus* was found on the gill while *Gyrodactylus sp.* was found on both the gill and skin. This could be because parasites of the *Dactylogyrus* genus are oviparous and infect mainly the gills of their hosts, while the *Gyrodactylus* members are viviparous and infect the skin of their host fish [21].

Our results showed the presence of the crustaceans, in particular, copepods parasites in CC located in different areas of the carp body: skin, gills and fins. The infection rate was 13.2%. Thus, our results support previous studies which reported the presence of this taxa in carp in Iraq [12, 4]. Several species of crustacean are known to infect fish farms in Iraq [11], however, only one species—*Argulus foliaceus*, was observed on the skin of three carp species, including the *C. carpio* [22]. Moreover, the presence of this copepod was associated with 11% of carp deaths.

Only one Cestode was identified in the intestine of the carps (*Bothriocephalus sp.*). The intestine as a habitat is preferred by the Cestodes; indeed, most tapeworms dwell in the intestine of their hosts, attached by suckers, hooks or other holdfast organs [23]. These are widespread parasites which develop into mature adults in the intestine of homeothermic animals. This parasite genus is behind the most important fish-borne zoonoses caused by a Cestodes parasite [24]. In our study, we found that the carps were infected at a 4.4% rate, this infection rate was associated with 18% deaths observed in CC.

The last group identified in the *C. carpio* ectoparasites in our study was leeches (*Annelida, Hirudinea*), with a 2% infestation rate. The literature mentions several species that affect different freshwater fishes in Iraq [25, 26]. Indeed, in Iraq, six taxa of leeches have been so far documented [25]. These ectoparasite was reported first in Iraq from the skin of fish species in ponds near Baghdad [27]. However, our results showed them in the gills of the carps. Moreover, this parasitic group has been associated with around 10% of carp deaths. This observation is supporting by some studies which have reported leaches as the cause of death in fishes [27, 28, 29].

Finally, we state that these parasites can quite often negatively impact the health and mortality of CC [18]. Indeed, during our study, we observed different signs in the infected fish. For example, we observed necrotic areas, ulcerations, and hemorrhaging on the gills and other body parts infected with both Protozoa and Monogeneas, while inflammation and mechanical injury were more frequently observed in fishes infected with crustaceans, tapeworms, nematodes and leeches. Our results support previous studies [7, 30], which reported important harmful effects of parasites on their hosts. Thus, the lesions or different signs observed in the fish could be due to the different mechanical pathways used by the parasites to better

colonize or attach to their host, which has mechanical and/or physiological consequences and production of enzymes and other substances that reduce the weight and growth and reproduction rates [30, 31]. Thus, it is important to carry out monitoring of fish systems in aquacultures, in order to better control parasitic infections and avoid loss of fish. The surveillance of CC from the Erbil aquacultures has allowed us to use some products (formalin, PP, and fenbendazole) for treatment against different observed parasites, and these products have proven to be very effective against the majority of the identified parasites. However, additional studies must focus on identifying fish parasites and their burdens and evaluating the pathological impact of parasites, such as *Capillaria* isolated from fish gills.

Carp fish in and around Erbil city were found to carry heavy parasitic infections, causing pathological and mechanical injuries in fish. These effects were associated with high mortality rates in fish. *Dactylogyrus sp.* was the most prevalent parasite affecting the *C. carpio*. The unexpected presence of the *Capillaria sp.* was observed for the first time infecting the gills. This result has not been recorded in any earlier studies in Iraq.

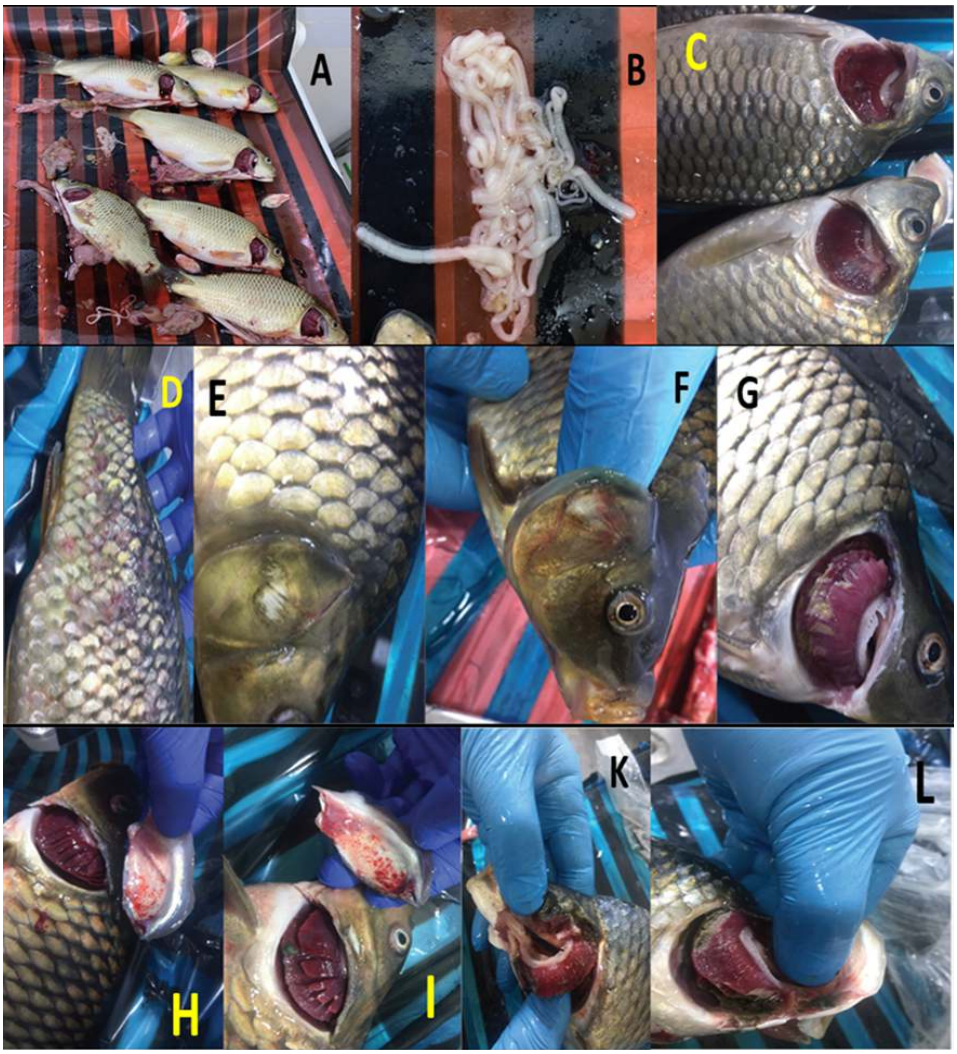

Figure 3
Clinical signs observed in infected fish. A-B) worm samples isolated from intestine, C) Pale whitened gills, D-G) Necrosis, mechanical injury, increased mucus decayed tissues, H-L) hemorrhage, necrosis, ulceration of infected gills

Materials & Methods

Samples collection

Research on the parasites was conducted from March to July 2018 on common carp fish from five farms, located around Erbil, North Iraq. Fish were collected using a bag net. All animals collected alive were kept in big, clean containers and transported to the laboratory for analyses. Identification of the fish was done using the Coad's list [13]. A total of 250 fish were collected for examination (average of 50 Carp per farm). Behavioral parameters such as sluggish movement, aggregation of fishes near oxygen sources, breathing difficulties, anorexia, lethargy, noting of dead fishes in early morning were recorded for each farm.

Examination

Initially, all the fish were visually examined. The aim of this step was to observe the worms, larva and other big parasites as well as all signs and symptoms on the gills and bodies of each fish, and the death rates were also recorded. Secondly, the microscopic (wet smear) analyzes were conducted on the mucus. The skin and the fins were gently scraped, and the scraped materials were placed on a clean microscopic slide and examined with a compound light microscope. For the gills, the bony arches were omitted and little filaments were cut and placed with saline on a slide, were covered by a coverslip on the slide and examined microscopically. The moving worms were obviously noted and photographed with a mobile camera.

Finally, we conducted internal examination of the fish. For this analysis, the body wall was cut from the ventral side, the intestinal parts were opened and examined for gastrointestinal parasite investigation, especially for Cestodes and Nematode worms. The detection and counting of intestinal parasitic eggs, larvae and cysts was performed using a compound microscope (objective 10X). We used the 40X and 100X objectives to take pictures. Identification of the parasites was based on stages morphology, as previously described in studies and guides [14,15,16]. Some locally available treatments were employed for the infected fish on the aquacultures and the mortality reduction was recorded.

Authors' Contributions

Experiment design: HM, NF, TM. Samples collection: HM, NF. Preparation of samples: NF, TM. Slides examination: HM NF. Analyzed the data: HM, NF, TM, LB. Research equipment (reagents, materials and analysis tools: HM, NF, TM. Wrote the paper: HM, LB.

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Competing Interests

The authors declare no conflict of interest.

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RESEARCH ARTICLE

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A role for GABA agonist in controlling the reproduction of female rats via hypothalamic ghrelin, kisspeptin, and RFRP-3 gene expression

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ABSTRACT

Kisspeptin stimulates gonadotropin releasing hormone (GnRH). The GnRH neurons receive inhibitory inputs from ghrelin, RFamide related peptide-3 (RFRP-3), and gamma-aminobutyric acid (GABA) neurons. Polycystic ovary syndrome (PCOS) is associated with increased levels of GnRH/LH and kisspeptin, and decreased release of GABA, ghrelin, and RFRP-3. In the present study, the effects of GABAB receptor agonist, baclofen, were investigated on *GnRH*, *KiSS1*, RFRP-3, and *ghrelin* gene expression in the hypothalamus of PCOS model rats. For induction of PCOS, female Wistar rats weighing 180-200g received intra-muscular injection of estradiol valerate. Fifteen PCOS rats in three groups received intraperitoneal injections of saline, 5, or 10 mg/kg baclofen for two weeks. The hypothalamic samples were dissected. Gene expression levels of *GnRH*, *KiSS1*, *RFRP-3*, and *ghrelin* were determined by real time qPCR method. Results revealed that baclofen significantly decreased the mean relative *KiSS1* gene expression compared to PCOS group. Also, the mean relative *RFRP-3* gene expression significantly increased in the baclofen-receiving rats in comparison to PCOS group. Furthermore, baclofen did not change GnRH or ghrelin mRNA levels in comparison to PCOS group. According to these results it can be concluded that in PCOS condition the GABAergic signaling pathway may suppress GnRH neural activity via down or up regulation of the intra-hypothalamic neuropeptides upstream of GnRH neurons.

Keywords

Baclofen, GnRH, kisspeptin, ghrelin, RFRP-3.

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Abbreviations

GnRH: Gonadotropin releasing hormone
RFRP-3: RFamide related peptide-3
GABA: Gamma-aminobutyric acid
PCOS: Polycystic ovary syndrome
LH: Luteinizing hormone
HPG: Hypothalamus - pituitary- gonadal
ARC: Arcuate nucleus
AVPV: Antero-ventral periventricular nucleus
POA: Preoptic area

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Introduction

Metabolic hormones regulate the normal release of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH). In addition to insulin resistance, the defects of hypothalamus - pituitary- gonadal (HPG) axis play a crucial role in the pathogenesis of polycystic ovary syndrome (PCOS) [1, 2].

Gamma-aminobutyric acid (GABA) suppresses the activity of GnRH neurons. Increased synthesis of GnRH has been shown in GABAB receptor knock-out female and male mice [3]. Baclofen inhibits the firing rate of GnRH neurons via hyper-polarization of them [4]. Using GABAB receptor antagonists completely neutralizes the inhibitory effects of baclofen on the firing rate of GnRH neurons [4]. It has been indicated that baclofen; GABAB receptor agonist, inhibits LH secretion [5]. The release of inhibitory neurotransmitters upstream of GnRH neurons such as dopamine and GABA are decreased in PCOS patients [6, 7].

Kisspeptin is a hypothalamic neuropeptide that is located in the arcuate nucleus (ARC) and antero-ventral periventricular nucleus (AVPV) of the hypothalamus. Kisspeptin acts upstream of GnRH neurons and conveys metabolic information to GnRH neurons [8, 9]. Kisspeptin/GPR54 signaling system regulates the HPG axis. Central or peripheral injection of the GPR54 receptor antagonist, peptide 234 blocks the stimulatory effect of kisspeptin on HPG axis activity [9]. The kisspeptin/GPR54 signaling system is one of the most important therapeutic targets to stimulate GnRH/LH release [8, 9].

RFamide related peptide-3 (RFRP-3) is a hypothalamic neuropeptide whose neuron cell bodies are located mainly in the dorsomedial hypothalamic nucleus (DMN). The fibers of RFRP-3 neurons project to other hypothalamic nuclei especially the preoptic area (POA), antero-ventral periventricular nucleus (AVPV), and arcuate nucleus (ARC) [10]. It has been revealed that RFRP-3 hyperpolarizes GnRH neurons and inhibits GnRH and LH secretion [10, 11].

Ghrelin, is an orexigenic peptide that is produced in the hypothalamus, stomach, and other peripheral organs [12, 13]. Ghrelin inhibits GnRH/LH and testosterone secretion [12, 13]. The GnRH neurons receive direct or indirect inputs from ghrelin neurons [13]. *Ghrelin* down-regulates *KiSS1* gene expression and decreases the stimulatory effects of kisspeptin on GnRH/LH release [14, 15]. In the present study, the effects of baclofen were investigated on hypothalamic *GnRH, KiSS1, RFRP3* and *ghrelin gene* expression in a rat model of PCOS.

Results

Mean relative *GnRH* gene expression did not significantly increase in the hypothalamus of PCOS rats in comparison to the control group (Figure 1). In PCOS rats that received 5 or 10mg/kg of baclofen, the mean relative *GnRH* gene expression did not significantly decrease in comparison to PCOS control group ($p \leq 0.05$, Figures 2 and 3).

The mean relative *KiSS1* gene expression increased significantly in the hypothalamus of PCOS rats compared to the control group ($p \leq 0.05$, Figure 1). In PCOS rats that received 5 or 10mg/kg of baclofen, the mean relative *KiSS1* gene expression significantly decreased in comparison to the PCOS control group ($p \leq 0.05$, Figures 2 and 3).

Induction of PCOS did not significantly decrease the mean relative hypothalamic *RFRP-3* gene expression compared to the control group (Figure 1). The mean relative *RFRP-3* gene expression significantly increased in PCOS rats that received 5 or 10mg/kg baclofen in comparison to PCOS control group ($p \leq 0.05$, Figures 2 and 3).

The mean relative *ghrelin* gene expression significantly decreased in PCOS rats in comparison to the control group ($p \leq 0.05$, Figure 1). The mean relative ghrelin gene expression did not significantly increase in PCOS rats that received 5 or 10mg/kg baclofen in comparison to the PCOS control group ($p \leq 0.05$, Figures 2 and 3).

Discussion

The obtained results showed that *GnRH* and *KiSS1* gene expression increased in the hypothalamus of PCOS rats. The results are in accordance with the literature and demonstrate that the higher GnRH/LH and kisspeptin levels are involved in the pathogenesis of PCOS [1, 16]. The increased kisspeptin neuronal activity leads to higher GnRH neuronal activity which results into excessive androgen secretion in PCOS patients [1, 16].

The present results showed that injection of baclofen significantly decreased the hypothalamic KiSS1 mRNA levels in PCOS model rats. Here we show the effects of baclofen on kisspeptin gene expression for the first time in PCOS condition. However, the present data are consistent with the previous studies that established an interaction between kisspeptin and GABAergic systems to control LH secretion. Both GABAA and GABAB receptor subtypes are expressed in kisspeptin neurons. Injection of baclofen hyperpolarizes the kisspeptin neurons and disturbs the surge secretion of GnRH/LH [17, 18]. Also, GABA release decreases in the PCOS conditions [6, 7] and the GABA- transaminase enzyme that degrades GABA,

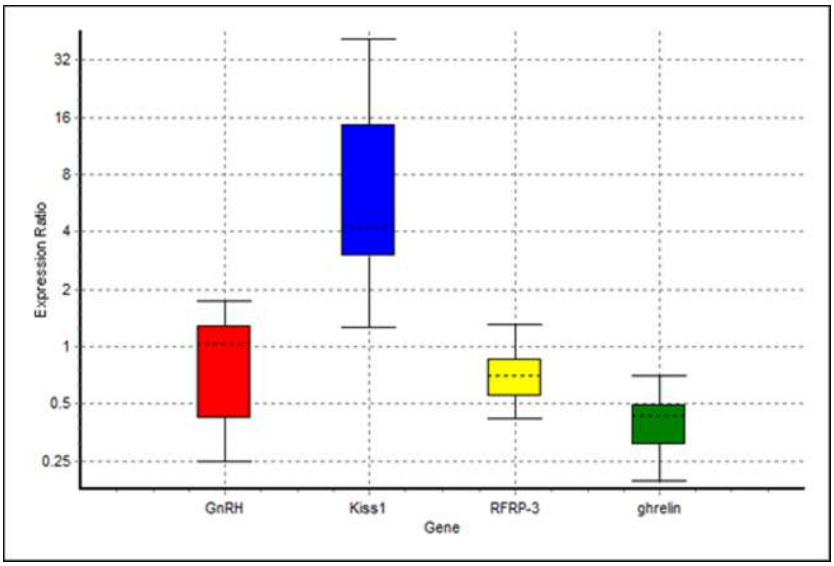

Figure 1.
The mRNA fold change of *GnRH*, *Kiss1*, *RFRP-3*, and *ghrelin* genes in PCOS rats in comparison to intact control rats. The cDNA amplified from GAPDH mRNA (as reference gene) was used to normalize the data. The significance difference was defined by $p \leq 0.05$.

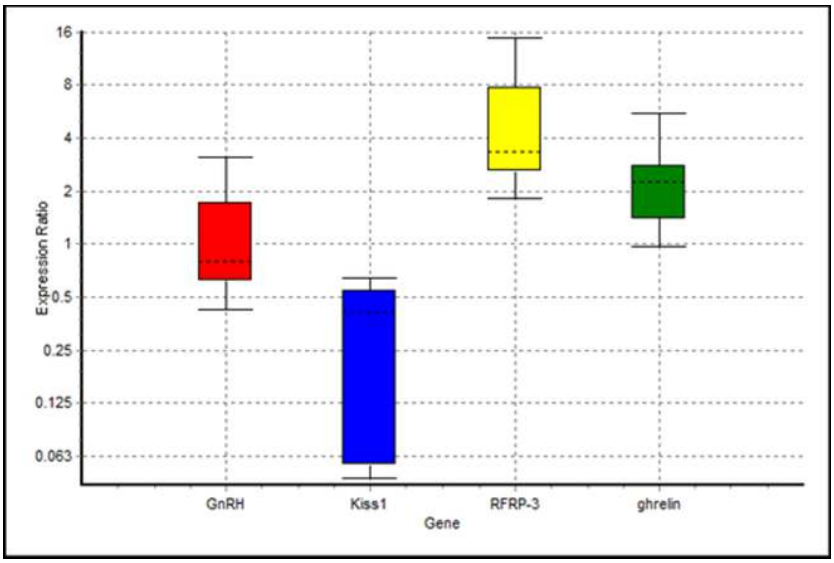

Figure 2.
The mRNA fold change of *GnRH*, Kiss1, *RFRP-3*, and *ghrelin* genes in PCOS rats receiving 5mg/kg baclofen in comparison to PCOS rats. The cDNA amplified from GAPDH mRNA (as reference gene) was used to normalize the data. The significance difference was defined by $p \leq 0.05$.

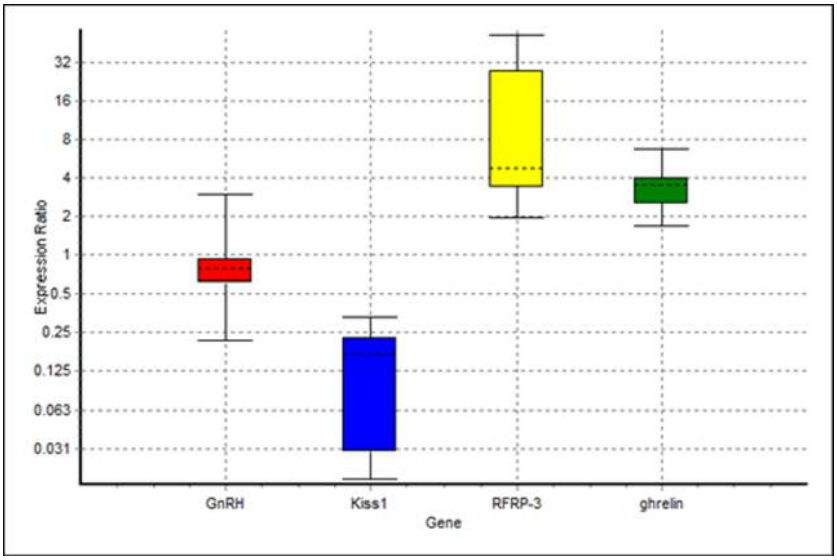

Figure 3.
The mRNA fold change of *GnRH*, *Kiss1*, *RFRP-3*, and *ghrelin* genes in PCOS rats receiving 10mg/kg baclofen in comparison to PCOS rats. The cDNA amplified from GAPDH mRNA (as reference gene) was used to normalize the data. The significance difference was defined by $p \leq 0.05$.

is significantly increased in the hypothalamus and pituitary of PCOS rats in comparison to the control group while glutamic acid decarboxylase enzyme that converts glutamate into GABA, is decreased in the hypothalamus and pituitary of PCOS rats [2]. Previous studies indicated that kisspeptin abolishes the inhibitory effects of baclofen on GnRH neurons [4]. Also, the administration of baclofen following kisspeptin attenuates the excitatory influences of kisspeptin on the depolarization of GnRH neurons [5]. Increased KiSS1 mRNA levels were observed in the arcuate nucleus (ARC) of GABAB receptor knock-out mice [3]. So, in this study, the decreased hypothalamic KiSS1 mRNA levels by baclofen may be a possible mechanism for the decline of GnRH synthesis in PCOS rats.

To find mechanisms involved in the regulatory effects of baclofen on *kisspeptin* gene expression, this study investigates the effects of baclofen on intra hypothalamic neuropeptides such as ghrelin and RFRP-3, both acting upstream of kisspeptin and GnRH neurons. The obtained results revealed that baclofen exerts a stimulatory effect on ghrelin mRNA levels in PCOS conditions. The results are in line with the previous studies and demonstrate an interaction between GABAergic, ghrelin, and kisspeptin signaling pathways. According to the previous results, ghrelin decreases GnRH/LH secretion and KiSS1 mRNA levels in the hypothalamus and pancreas [14, 15] while baclofen increases the plasma ghrelin concentration [19]. So, increasing hypothalamic ghrelin mRNA levels may be a contributing factor for baclofen to decrease *KiSS1* gene expression in PCOS rats.

Our results demonstrated that the PCOS condition did not cause a significant decrease in hypothalamic *RFRP-3* gene expression in comparison to control rats. This is in contrast to the findings of Shaaban et al. that demonstrated a significant decrease of RFRP-3 mRNA levels in dorsomedial hypothalamic nucleus [20]. Maybe this conflict could be to the used method for induction of PCOS model rats. Herein estradiol valerate was used for induction of PCOS, while Shaaban et al. used constant light induction to generate PCOS model [20]. However, further studies are needed for evaluation of the *RFRP-3* gene expression in PCOS conditions. Interestingly, our results indicate the stimulatory effects of baclofen on *RFRP-3* gene expression in PCOS rats. As previously shown, the RFRP-3 suppresses the GnRH/LH secretion [21] and there is a reverse relationship between RFRP-3 and kisspeptin function [21]. The RFRP-3 receptor (GPR147) is expressed in kisspeptin neurons located in ARC and AVPV nuclei of hypothalamus and RFRP-3 fibers project to kisspeptin neurons [22, 23]. For interpretation of the obtained results, it can be suggested that the increase of RFRP-3 mRNA levels after baclofen injections might play an important role in suppressing kisspeptin and GnRH neural activity. To better understand the action of GABAergic system on controlling HPG axis activity in PCOS conditions, it is suggested that further studies should try to investigate the effects of intra cerebral ventricular injection of baclofen or other GABA agonists on gene expression levels of ovarian or intra hypothalamic peptides upstream of GnRH neurons.

In conclusion, polycystic ovary syndrome (PCOS) is associated with increased mRNA levels of hypothalamic kisspeptin which stimulate the activity of hypothalamus- pituitary- gonad (HPG) axis. However, mRNA levels of inhibitory neuropeptides upstream of GnRH neurons such as ghrelin decreased in the hypothalamus of PCOS rats. Our results demonstrated that the intraperitoneal injections of baclofen, significantly decreased KiSS1 mRNA levels in the hypothalamus of PCOS rats. Baclofen exerts stimulatory effects on hypothalamic ghrelin and RFRP-3 mRNA levels in the hypothalamus of PCOS rats. The obtained results suggest that GABAergic signaling pathway is involved in the controlling of HPG axis activity to some extent by down- or up-regulation of the hypothalamic stimulatory and inhibitory neuropeptides such as kisspeptin, ghrelin, or RFRP-3 in PCOS patients.

Materials & Methods

Animals

In this study, 20 female Wistar rats weighing 180-200 g (provided by the Iran University of Medical Sciences) were housed in the cages under controlled temperature (22 ± 2 °C) and light (12h light/ dark cycle). All procedures for the maintenance and the use of experimental animals were approved by the research and ethical committee of Ardabil University of Medical Sciences (code: IR.ARUMS.REC.1398.511).

Induction of polycystic ovary syndrome

The vaginal smear was performed for two consecutive weeks to select the rats with the normal estrus cycle. In the estrus stage which was characterized by cornfield epithelial cells, 15 rats received an intramuscular single dose of 2 mg/rat estradiol valerate (Aburayhan Co., Iran) dissolved in 0.2 ml sesame oil (Barij Essence Co., Iran). Five rats in the estrus stage received a single intramuscular injection of 0.2 ml sesame oil as an intact control group. Sixty days after the estradiol valerate injection, the polycystic status was confirmed by observation of persist cornfield epithelium cells with vaginal smear.

Intraperitoneal injections

Fifteen PCOS rats in three groups received intraperitoneal injections of saline, 5, or 10 mg/kg baclofen (Zahravi Co., Iran) in a volume of 0.2 ml at 9:00-9:30 for two weeks. Also, five intact rats received 0.2ml saline as a control group for two weeks.

Microdissections and real-time polymerase

chain reaction (RT-PCR)

One day following the last injection, animals were anesthetized by injection of ketamine and xylazine. The hypothalamic samples were dissected. According to coordinates of the Paxinos and Watson Atlas, the brains were placed ventral side up, and anterior coronal slices were cut from 1 mm anterior to optic chiasm. The slices were dissected laterally up to the hypothalamic sulci, and posterior coronal slices were cut posterior to the mammillary bodies [24, 25]. Hypothalamic samples were stored at -80 °C. Total RNA was isolated from individual frozen samples using the acid guanidinium thiocyanate-phenol-chloroform extraction method.

To synthesize the first-strand cDNA, 5µg total RNA, 1µl of 100 µM Oligo(dT)$_{18}$ primer, 4µl of 5X Reaction Buffer, 1µl of RiboLock RNase Inhibitor (20 U/µl), 2µl of 10 mM dNTP Mix, 1µl of RevertAid RT (200 U/µl), and nuclease free water in a volume of 20 µl were incubated at 42 °C for 60 min and the reaction was terminated by heating at 70 °C for 5min (Thermo Scientific RevertAid RT reverse transcription kit, USA). Changes in gene expression levels were determined by using Rotor Gene 6000 (Corbette, Germany) and SYBR Green I kit (Takara Bio Inc., Japan). The PCR cycling conditions were as following: first denaturation 95 ºC for 2 min, followed by 40 cycles of denaturation at 95 ºC for 5 sec, annealing at 60 ºC for 20sec (*KiSS1, RFRP-3, GnRH* or *GAPDH*), annealing at 54 ºC for 20 sec for *ghrelin* and extension at 60 ºC for 25 sec. Specific oligonucleotide sequences for forward and reverse primers are shown in Table 1. The GnRH, KiSS1, RFRP-3, ghrelin and GAPDH amplified products were 133, 98, 93, 132, and 120 base pairs, respectively. In this study PCR efficiency of each gene was calculated using LinRegPCR software. Based on the outputs derived from LinReg PCR software, the PCR efficiency for GAPDH, ghrelin, GnRH, Kiss1 and RFRP-3 were 2.06, 1.762, 2.041, 1.78 and 1.846, respectively.

Table 1.
Sequences of forward and reverse primers used in this study.

Gene	primers sequences
GnRH (NM_012767)	F: 5'-GCCGCTGTTGTTCTGTTGACTG-3'
	R: 5'- CCTCCTCCTTGCCCATCTCTTG-3'
KiSS1 (NM_181692)	F: 5'- TGATCTCGCTGGCTTCTTGGC -3'
	R: 5'- GGGTTCAGGGTTCACCACAGG-3'
RFRP3 (NM_023952)	F: 5'- GAGTCCTGGTCAAGAGCAAC-3'
	R: 5'-ACTGGCTGGAGGTTTCCTAT -3'
Ghrelin (NM_021669)	F: 5'- AATGCTCCCTTCGATGTTGG -3'
	R: 5'-CAGTGGTTACTTGTTAGCTGG -3'
GAPDH (XM_039103945)	F: 5'- AAGAAGGTGGTGAAGCAGGCATC -3'
	R: 5'-CGAAGGTGGAAGAGTGGGAGTTG-3'.

Statistical analysis

The data were analyzed by using REST 2009 software. In all cases, the significance was defined by $p \leq 0.05$.

Authors' Contributions

F.M. and H. KH. conceived and planned the experiments. E.R.R. and F.M. carried out the experiments. F.M., H.KH., E. R.R., A.A., and M.GH. contributed to the interpretation of the results. F.M. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, data analyses, and the manuscript.

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Competing Interests

The authors declare no conflict of interest.

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Evaluation of hormonal treatments for different scenarios of cystic ovarian follicles in dairy cattle

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ABSTRACT

The present study aimed to evaluate the efficacy of different hormonal interventions in the treatment of cystic ovarian follicles (COF) based on different scenarios, including the size of the cyst and the presence of other follicles on the ovaries of dairy cows. A total of 199 Holstein cows with COF in the first 100 days postpartum were enrolled in the study. These cows were randomly assigned to the four following groups: 1) GnRH (G) group: intramuscular (IM) injections of 100 µg gonadorelin acetate on day 0 and 150 µg d-cloprostenol 7-12 days later, 2) double GnRH (DG) group: two IM injections of 100 µg gonadorelin acetate at 6 h intervals on day 0 and d-cloprostenol 7-12 days later, 3) intravaginal progesterone device (IPD) group: insertion of PRID Delta for 7-12 days and injection of d-cloprostenol on the withdrawal of PRID Delta, and 4) control group: IM injection of 2 mL sterile saline on day 0 and 7-12 days later. The cure rate of COF significantly improved in the G and DG groups, in comparison with the IPD and control groups. There was no significant difference between the cows in the G and DG groups. In the control group, animals with ovarian cysts smaller than 2.5 cm had a significantly greater self-cure rate, compared to the other cows. In conclusion, this field study demonstrated a good clinical cure in the groups of cows treated by GnRH. However, no improvement was observed in the reproductive performance of these animals.

Keywords

dairy cattle; GnRH; progesterone; cystic ovarian follicles

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Abbreviations

COF: Cystic ovarian follicle
GnRH: gonadotropin-releasing hormone
LH: luteinizing hormone
P4: progesterone
PGF2α: Prostaglandin F2α
IPD: Intravaginal progesterone device
BCS: body condition score
DIM: Days in milk.

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Introduction

Cystic ovarian follicles (COFs) have been defined as follicles of at least 2 cm in diameter on one or both ovaries without any active luteal tissue that disrupts normal ovarian function. The COF, as a reproductive disorder, is a serious economic problem with a high incidence rate ranging from 16.3% to 30.3% during the first 9-10 weeks post-partum in dairy cows worldwide [1, 2, 3]. In a study by Sakaguchi et al., 80% of COF cases were detected before the first ovulation and 20% before the second or third ovulation [4, 5].

Cysts developing from pre-ovulatory follicles, which are unable to ovulate, persist and interfere with normal ovarian function [6]. The COF results from a "hormonal imbalance" within the hypothalamic-pituitary-gonadal axis. The defective surge of gonadotropin-releasing hormone (GnRH) by the hypothalamus occurs due to impaired response to follicular estradiol [6]. This failure in GnRH surge leads to the decreased stimulation of the anterior pituitary gland for surging pre-ovulatory luteinizing hormone (LH). This hypothalamic insensitivity to estradiol may result from the intermediate concentration of progesterone (P4), which is common in cows with COF [7, 8, 9, 10, 11, 12]. Moreover, it has been shown that higher milk production in dairy cattle is associated with an increased risk of ovarian cysts [4]. Several hormonal and metabolic modifications that are associated with negative energy balance can affect the function of the ovaries. However, the exact underlying mechanism of ovarian cysts is still unclear [2].

Although the efficacy of different hormonal treatments has been evaluated in various studies, no criteria have been considered for treatment decisions. Therefore, given the high treatment costs, it seems necessary to adopt targeted selective treatments. It seems that the concurrent ultrasonographic examination of ovaries with conventional therapies, such as GnRH administration, intravaginal P4 devices, and hormonal combinations may eliminate some of the ambiguity in findings.

Under field conditions, GnRH injection is the treatment of choice. The GnRH and its analogs have been considered for COF treatment with variable success rates irrespective of the type of the cyst, lack of antigenic effects, and cost of the treatment [6,13,14]. Following GnRH treatment, LH level reached a peak in 2 h and remained high in the blood for 6 h, while normally, the LH concentration remains elevated for 12 h [15]. In general, three weeks after GnRH administration, a resumption of the normal estrous cycle is observed in 60%-95% of the treated cows followed by the pregnancy rates of 60%-85%. Furthermore, COF can be treated by intravaginal P4-releasing devices. The success of this method is high, as 70%-85% of cystic cows recover within two weeks [16]. Prostaglandin F2α (PGF2α) or its analogs are used to treat luteal cysts [17]. In addition, this treatment can be combined with GnRH/hCG to treat follicular cysts and reduce the duration of the luteal phase [18]. Various sources have provided information on the risks following the use of hormonal products in livestock and their impact on health, fertility, and the livestock economy. Consequently, a proper and minimal selection of hormones is required for treating various conditions, such as ovarian cysts [19].

With this background, this study aimed to evaluate the success of different treatments, including GnRH and intravaginal P4 devices for ovarian cysts based on distinct ovarian structures and the size of ovarian cysts by using ultrasound examination. Moreover, we compared the efficacy of conventional treatments with two doses of GnRH injected at an interval of 6 h.

Results

Out of 221 Holstein cows, 199 cows were included in the present study. Twenty-two cows were excluded from the study, due to high progesterone concentration (> 1 ng/ml) at the first ultrasonographic exam. At the beginning of the study there were no significant differences in the DIM, parity, milk yield, and BCS of different groups. Moreover, the treatment groups did not have a significant difference in terms of the size of the cyst and follicle.

The cure rate was significantly different between the test and control groups ($p = 0.0001$). The cure rate of the ovarian cyst was significantly higher in the G and DG groups, compared to the PRID Delta group ($p < 0.05$) (Table 1). No significant difference was found between cows that received single or double injections of GnRH.

Table 1.
Total cure rates in treatment groups.

Group	N	%Cure rate (n)
G	49	83.67 (41)^{a}
DG	50	82.00 (41)^{a}
IPD	48	64.58 (31)^{a,b}
C	52	46.15 (24)^{c}

G: GnRH, DG: Double GnRH, IPD: PRID Delta, C: Control; different superscripts in columns indicate significant differences ($p < 0.05$).

Cyst size significantly affected treatment outcomes in all test groups ($p < 0.05$) (Table 2). The results showed that 53 out of 68 cows (77.94%) with a COF diameter between 2 to 2.5 cm, and 84 out of 131 animals (64.12%) with a COF diameter >2.5 cm were treated.

Table 2.
Total cure rates based on cyst size.

Cyst size (cm)	N	%Cure rate (n)
≤2.5	68	77.94 (53)[a]
>2.5	131	64.12 (84)[b]

Different superscripts in columns indicate significant differences ($p < 0.05$).

In cases with cysts greater than 2.5 cm in diameter, there was no statistically significant difference among treatment groups. However, in the DG group, the cure rate was numerically higher. The cure rate of cysts ≤2.5 and >2.5 cm in diameter was not significantly different between treatment groups. In the control group, cows with ovarian cysts ≤2.5 cm had a significantly higher self-cure rate compared to other cows ($p < 0.05$) (Table 3).

There was no significant difference between the groups, regarding cure rate and follicular size. In the control group, the cure rate was significantly greater in cows with follicular size ≥1 cm in diameter, compared to animals with smaller follicles ($p < 0.05$) (Table 4).

Table 3.
Percent of cure rates based on cyst size in different groups.

Group	N	Cure rate (%)	
		Cyst size ≤ 2.5cm	Cyst size >2.5cm
G	49	21/23 (91.30%)	20/26 (76.92%)[a]
DG	50	11/13 (84.62%)	30/37 (81.88%)[a]
IPD	48	10/16 (62.50%)	21/32 (65.63%)[a]
C	52	11/16 (68.75%)	13/36 (36.11%)[b]

G: GnRH, DG: double GnRH, IPD: PRID Delta, C: control; different superscripts in columns show significant differences ($p < 0.05$).

Overall, the difference in the treatment response, including the ovulation of the cyst, ovulation of the follicle, and luteinization or regression of the cysts was significant among treatment groups, ($p < 0.05$) (Table 5). The first service conception rate and the percentage of pregnant cows on 120 DIM were not significantly different among the groups (Table 6).

Table 4.
Total cure rates based on follicular size.

Group	N	Cure rate (%)	
		Follicular size < 1^{cm}	Follicular size ≥1^{cm}
G	49	19/22 (86.36%)	22/27 (81.84%)
DG	50	15/19 (78.95%)	26/31 (83.87%)
IPD	48	19/26 (73.08%)	12/22 (54.55%)
C	52	4/20 (20.00%)[a]	20/32 (62.50%)[b]

G: GnRH, DG: double GnRH, IPD: PRID Delta, C: control; different superscripts in the rows show significant differences ($p < 0.05$).

Discussion

In the present study, different hormonal interventions were evaluated for treating COF based on varying scenarios that occurred on the ovaries of dairy cows. Cyst wall diameter evaluated by ultrasound was highly correlated with milk P4 concentration with an accuracy range of 75%–95% as reported by various authors [20, 16, 21, 22]. The cows in G, DG, and IDP groups showed significantly better results than the control group. The overall treatment results in the G and DG groups were significantly better than the IPD group. GnRH is used to induce a new follicular wave, luteinize the cyst, and/or ovulate the follicle at the same time [23]. GnRH induces LH release leading to the highest plasma LH concentration in 90-150 min, which initiates the formation of active luteal tissue as shown by elevated serum P4 levels 7 days post-treatment [24, 25]. The concentration of blood LH depends on the dose of injected GnRH and reaches the peak level in 2 h after injection. The LH concentration decreases after 4 h. Based on reports, the range of LH surge is directly related to the success of treatment [25].

A success rate of 72% was observed by applying GnRH as the treatment, while the average cyst diameter was 2.5 cm and the cysts did not ovulate after GnRH injection [6]. In another study, ovarian cysts were treated with the Ovsynch program causing a new follicular wave in 100% of the cases due to GnRH injection, and a dominant follicle formed and ovulated in 83% of cases [1]. However, the efficacy of GnRH treatments is controversial in different studies and in some studies, there was no difference in ovarian response between treated and untreated animals [26].

Dinsmore et al. showed that the concomitant injection of GnRH and PGF2α have no advantage over GnRH alone [27], while another study demonstrated that cows received GnRH alone, experienced a higher rate of adverse effects [21].

In a study by Kawate (2011), the simultaneous

Haddadi et al. IJVST 2021; Vol. 13, No. 1
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Table 5.
Treatment response (ovulation of the cyst, ovulation of the follicle, and luteinization or regression of the cysts) in different treatment groups.

Group	Cured (n)	% Ovulation of the cyst (n)	% Ovulation of the follicle (n)	% Luteinization or regression (n)
G	41	58.54[a] (24)	24.39[a] (10)	17.07[a] (7)
DG	41	43.90[a] (18)	41.46[a] (17)	14.63[a] (6)
IPD	31	22.58[b] (7)	35.48[a] (11)	41.93 (13)
C	24	16.67[b] (4)	66.67[b] (16)	16.67[a] (4)

G: GnRH, DG: double GnRH, IPD: PRID Delta, C: control; different superscripts show significant differences ($p < 0.05$).

Table 6.
First service conception rate and percentage of pregnant cows on 120 DIM in different treatment groups.

Group	N	FSCR % (n)	% Pregnant cows on 120 DIM (n)
G	46	36.96 (17)	58.69 (27)
DG	48	37.50 (18)	56.25 (27)
IPD	47	44.68 (21)	61.70 (29)
C	50	34.00 (17)	56.00 (28)

G: GnRH, DG: double GnRH, IPD: PRID Delta, C: control

application of controlled internal drug release with GnRH was evaluated in a 9-day protocol. The results of this study showed no advantage for the combination of intravaginal P4 and GnRH in treating ovarian cysts, compared to GnRH alone as an Ovsynch protocol [20].

The cure rate of the cysts using GnRH in this study was similar to other studies. In most studies, the effect of GnRH on the treatment of ovarian cysts was assessed based on two criteria, ovulation of a follicle in the presence of the cyst, and/or luteinization of the cyst wall. In this study in addition to these criteria, another therapeutic response was observed. In 53 cases out of 137 treated cows (38.68%), the cyst ovulated. In 40 out of 53 cases, cyst diameter at the time of treatment was ≤ 2.5 cm and ovulation of the cysts was significantly higher in cases with a cyst diameter of ≤2.5 cm. The ovulation of COF was observed in all treatment groups and was the most observed treatment response in G and DG groups (58.54% and 43.9%, respectively). The difference between the results of this study and those of others might be due to the lack of the ultrasonographic evaluation of ovarian structure during treatment and follow-up in some studies, the difference in therapeutic responses, and the variations in ovarian cyst definition.

Ovarian cysts previously were defined as follicular structures >2.5 cm in diameter, while in recent definitions, cysts are known as follicles with diameters >2 cm, even >1.7 cm [3, 28].

A study on the effect of PRID Delta in the treatment of ovarian cysts found that 83% of cows with follicular cysts responded to treatment within 14 days of starting treatment and developed a corpus luteum [29]. In the current study, the cure rate after treatment with PRID Delta was 64% which was significantly higher than the control group and lower than the G and DG groups, and ovulation of the follicle was the most treatment response in this group.

The P4 implant treatment may disrupt the endocrine environment required for maintaining COFs leading to their regression [30]. The P4 acts against follicular cysts by restoring hypothalamic responsiveness to estradiol-positive feedback resulting in normal estrus and ovulation seven days after implant removal.

This study aimed to make more specific therapies based on the characteristics of the ovarian structures, such as cyst and follicular size at the time of treatment. A higher cure rate was observed in G and DG groups, in comparison with the animals in PRID and control groups irrespective of the cyst size and presence or absence of follicle >1 cm in diameter. It was shown that GnRH was the most effective and the best choice for the treatment of cysts of any size. The cure rate for cysts ≤2.5 cm in diameter was higher in all groups. In the control group, the self-cure rate of cystic structures was 46.15% (24/52) and the self-cure rate of ovarian cysts ≤2.5 cm in diameter was significantly higher than that of ovarian cysts >2.5 cm in diameter. So it was better not to treat the cows with cysts ≤2.5 cm in diameter prior to 50 DIM, which concomitantly had follicle(s) >1 cm on their ovaries because the self-cure rate was very high in these cases (63.63%). It may be due to the inactivity of follicular cysts, which do not produce estrogen and the new follicle was allowed to grow up to 1 cm in diameter.

Morrow et al. (1969) showed that the self-cure rate of the cysts that developed within 45 days postpartum was about 50% [31]. In a study by López-Gatius (2002), 80% of cows with ovarian cysts in their first lactation were spontaneously cured, while this rate was 30% for older cows [21]. In the present study, the spontaneous cure rate was not significantly different between the primiparous and multiparous dairy cows (40% and 47.61%, respectively).

No significant improvement in reproductive performance (first service conception rate and percentage of pregnant cows on 120 DIM) was observed among different groups. This may be due to various individual and environmental factors that affect the success of different hormonal therapy and fertility [32, 33, 14].

Materials & Methods

Animals and Study Design

The experiment was conducted in a dairy herd with approximately 900 lactating Holstein cows in Mashhad, northeast of Iran. The sample size was calculated based on the study performed by Kim et al. [35], Cows were milked thrice daily with average daily milk production of about 38 kg. The animals were housed in free-stall barns with sand bedding and were fed a total mixed ration. The voluntary waiting period in this herd was 45 days. A total of 199 Holstein cows up to 100 days in milk (DIM) with ovarian cysts were enrolled in the present randomized controlled trial.

Cystic cows were identified after the first ultrasound examination (real-time linear array, 7.5 MHz rectal probes, Easi-Scan, Scotland, UK). In order to confirm the diagnosis, a second ultrasound scan was performed 7-12 days after the first one. Cows with follicles larger than 2 cm in diameter in the absence of corpus luteum in both examinations were identified as COF. Finally, the treatments were carried out for 199 cows. The pattern of ovarian structure, including the size and number of cysts and follicles in the ovaries, was assessed in the second ultrasound scan. Afterwards, the cows were randomly assigned to the four following groups: 1) GnRH (G) group: an IM injection of 100 µg gonadorelin acetate (Gonasyl; Syva, Spain) on day 0 and 150 µg d-cloprostenol (Luteosyl; Syva, Spain) 7-12 days later, 2) double GnRH (DG) group: two IM injections of 100 µg gonadorelin acetate with 6 h interval on day 0 and d-cloprostenol 7-12 days later, 3) intravaginal progesterone device (IPD) group: (PRID Delta, Ceva, France) insertion for 7-12 days followed by PRID removal and simultaneous d-cloprostenol injection, and 4) control group: two IM injections of 2 mL sterile saline on days 0 and 7-12.

The third ultrasound examination was performed 7-12 days after the initiation of treatment in G, DG, and control groups. In the IPD group, the third examination was carried out 7-12 days after PRID removal. In all examinations, changes at the ovarian level were recorded in detail. The cases were considered as treated based on a set of criteria, including the presence of corpus luteum, cyst wall luteinization, cyst regression, and estrus following PRID Delta removal.

Other inclusion criteria in the current study entailed not being affected by concomitant diseases, such as lameness, endometritis and the lack of other concurrent ovarian problems, like adhesion and tumor. At the time of enrolment, the body condition score (BCS) of cows was recorded by a single evaluator based on a scale of 1 to 5 in increments of 0.25 [34]. Data on milk yield was utilized to assess the effects of milk yield on different treatments.

Milk Samples and Laboratory Analysis

Milk samples were collected into 9-mL evacuated tubes at the time of enrolment (on the day of cyst diagnosis) and on the day of PGF2α injection in the 1st and 2nd treatment groups and 7-12 days after PRID removal in the 3rd group and on the day of the last injection in the control group. The milk samples were chilled on ice packs immediately after collection, frozen at -20°C within 2 h, and delivered to the laboratory for further analysis.

Progesterone concentration of milk samples was measured using a solid-phase radioimmunoassay commercial kit (Progesterone RIA KIT; Institute of Isotopes Co. Ltd.; Hungary) by a RALS Gamma Counter instrument (Dream Gamma-10; Shinjin Medics Inc.; South Korea) according to the guidelines of the manufacturer [7]. Cases with milk P4 concentration of 2 ng/mL were considered to have active luteal tissue. Ultrasound scan and P4 concentration were used to assess therapeutic response on the day of PGF2α injection. Presence of a corpus luteum and luteinization or regression of the cyst are considered as criteria for successful treatment. The presence of luteal tissue was confirmed by measuring P4 concentration in milk concurrent with the third ultrasonic examination in G, DG, and control groups. However, the measurement of P4 concentration in the IPD group was performed 7-12 days after PRID removal.

Data Management and Statistical Analysis

All analyses were performed using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). The relationship between treatment regimens and different outcomes, including cure rate, percentage of pregnant cows by 120 DIM, first service conception rate, cyst size, and follicular size were analyzed by using Chi-square test with PROC FREQ. Parity, BCS, DIM, and milk production were considered as covariates in the models. The variables were removed by manual backward stepwise elimination if $p > 0.2$. Finally, the relationships between the factors were assessed using multivariable logistic regression as PROC LOGISTIC modeling through a backward selection model. Cows that had a BCS of <3 were classified as thin, a BCS of 3.25 or 3.5 as ideal, and a BCS of > 3.75 as fat. Based on parity, the cows were classified into two groups of primiparous and multiparous cows. Dummy variables were created for DIM (1: DIM ≤ 45 and 2: DIM > 45), milk yield (1: ≤ 40 and 2: > 40), cyst size (1: cysts of ≤ 2.5 cm and 2: cysts of

Haddadi et al. IJVST 2021; Vol. 13, No. 1
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> 2.5 cm), and follicular size (1: follicles of ≤1 cm and 2: follicles of > 1 cm). To determine the degree of relationship between the risk factors and outcome variables, the odds ratio, and 95% confidence interval were calculated. For all statistical analyses, $p < 0.05$ was considered significant.

Authors' Contributions

N.F., H.S. and M.H. conceived and planned the experiments. M.H. and N.F. carried out the experiments. H.S., M.H. and N.F. contributed to the interpretation of the results. M.H. and N.F took the lead in writing the manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Molecular identification and phylogenetic analysis of *Chlamydophila abortus* isolated from sheep and goats

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ABSTRACT

Chlamydophila abortus is one of the major causes of pregnancy failure (abortion) in sheep and goats in many countries. In the present study, milk samples from sheep and goat herds of West Azerbaijan, Iran were examined for C. *abortus* using PCR and nucleotide sequencing. A total number of 360 milk samples were randomly collected from sheep (n=180) and goats (n=180) of three different regions of West Azerbaijan province during 2018. DNA was isolated from samples and the nested-PCR was employed targeting the *16S rRNA* gene for detection of *Chlamydia spp.* The *omp* gene was amplified and sequenced for the characterization of detected *C. abortus.*The results showed that 8.61% (95% CI: 6.13%–11.96%) of the examined samples (11.67% sheep and 5.56% goat milk samples) were positive for *C. abortus.* The frequency of positive samples in the central region was significantly higher than in other regions. Positive samples for *C. abortus* from animals with a history of abortion were significantly higher than those without a history of abortion. Positive samples in autumn were significantly higher than the other seasons and also, in animals more than four years old were significantly higher than other age groups. Sheep infection was significantly higher than the goats. Phylogenetic analysis based on the helicase gene showed that two sequenced isolates clustered closely with the other *C. abortus* isolates reported in the GenBank. In conclusion, small ruminants in West Azerbaijan province were contaminated with C. *abortus* and they could shed this organism into the milk.

Keywords

Chlamydophila abortus, omp gene, nested-PCR, helicase gene

Number of Figures: 5
Number of Tables: 2
Number of References: 49
Pages: 9

Abbreviations

C. abortus: Chlamydophila abortus
PCR: polymerase chain reaction
PZ: plasticity zone
OMP: outer membrane protein

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Introduction

The *Chlamydia psittaci* serotype 1 or *Chlamydophila abortus* is a non-motile, coccoid, obligate intracellular parasite from the family *Chlamydiaceae*. It has been recently given a new classification with 11 separate species. It is important as a causative agent of the reproductive system in a small ruminant [1]. *C. abortus* causes enzootic abortion in sheep, which is an infectious disease featured with placentitis and abortion. The complication decreases the breeding rate of sheep in different countries and it is not limited to sheep as it also affects goats and cattle [2-4]. The infected animals demonstrate no clinical signs until abortion or delivery of very weak lambs [2-4]. Normally, abortion happens during the last 2-3 weeks of pregnancy. There are reports that the abortion rate in one year age is low and increased to thirty percent at the age of two, followed by a 5-10% increase in the third year [5]. There are reports of latent infections for more than three years [6]. Pathological findings in this infection can begin in the third month of pregnancy. The finding in the placenta is exudate with yellow color, thickness in the membrane of cotyledons, and cotyledon color change to red [7].

To have a direct diagnosis, pathogen isolation, direct microscopic examination, serological tests, and DNA-based methods are used [8, 9]. Isolation of *chlamydia* in cell culture is not easy and time-consuming. In the CFT test, cross-reactions between *C. abortus* and other bacteria like *Acinetobacter* can be observed [4]. There has been a surge in the conventional and real-time PCR use to identify *C. abortus* in clinical samples. To this end, the PCR methods are used with amplification on the chlamydial outer membrane protein (*omp1, ompA,* and *omp2* genes), genes encoding 16S rRNA and helicase, the polymorphic membrane gene *pmp*, and the 16S-23S rRNA intergenic interval [10-12]. It is important to use rapid and reliable diagnostic tests for fast disease control. A highly sensitive method to find highly low copy number target DNA is nested-PCR. The nested-PCR method can find a variety of fastidious microorganisms with a significant increase in sensitivity and specificity [13].

While there are serological studies on *C. abortus* infection, our knowledge of the prevalence of *C. abortus* infections in sheep and goats' milk in Iran is very limited. To date, seven *C. abortus* genome sequences have been published [14, 15]. The UK strain S26/3 was the first reference genome, comprised of a 1.1 Mb chromosome and, unlike other *Chlamydia*, lacked any virulence-associated plasmid [16]. Two Greek isolates, LLG and POS, originating from the aborted fetuses of a goat and sheep respectively, represent the most diverse variant strains identified to date, with a further closely related strain recently described [17]. Two molecular typing schemes exist, using either multiple-locus variable-number tandem repeat analysis (MLVA), which has only identified seven MLVA sequence types (MTs) [18], or multiple locus sequence typing (MLST) [18], where only six MLST sequence types (STs) have been defined. This is in sharp contrast to greater diversity in other species of *Chlamydia* [19]. Studies on limited numbers of samples suggest that *C. abortus* isolates in livestock appear to be largely monomorphic: low diversity is observed throughout the genome, even within the plasticity zone [PZ], a region of high genomic variation in other chlamydial species [15, 16]. Infections of *C. abortus* in sheep and goats have generally been documented serologically in West Azerbaijan province, in the North West of Iran. However, studies on pathogen isolation and detection by PCR are rare in this region. Therefore, we conducted this study to isolate *C. abortus* in sheep and goats in West Azerbaijan province, Iran, using the nested-PCR technique. We also conducted a phylogenetic analysis to compare our isolates with other *Chlamydia* species that were deposited in GenBank based on partial helicase gene sequence.

Results

Amplification of 16SrRNA gene

Among 360 milk samples collected from sheep and goats, 31 samples (8.61%, 95% CI = 6.13–11.96) were positive for *chlamydia spp.* amplifying a fragment of 127 bp of the *16S rRNA* gene using nested-PCR. The prevalence of *Chlamydia spp.* in the milk of two examined species were statistically significant. The prevalence of *C. abortus* infection was significantly different in terms of regions with the highest frequency in the central region (Table 1). Animals with abortion history showed higher number of positive milk samples for C. abortus. In terms of seasonal prevalence, the highest number of positive samples for *C. abortus* was recorded in autumn. *C. abortus* was found in 17.24% of milk samples from animals more than 4-year-old, which was significantly higher than the other age groups (Table 1).

Amplification of ompA gene

Among 31 positive samples (*16S-rRNA* gene), all samples were positive with *C. abortus* amplifying a fragment of 479-bp of the *ompA* gene using PCR. In this study, only 31 cases (8.61%, 95% CI = 6.13–11.96) were identified as *C. abortus* in sheep and goats by producing a 479-bp fragment using PCR. (Figure 1).

Table 1.
The epidemiological characteristics associated with *C. abortus* prevalence in sheep and goats in West Azerbaijan province, Iran

Variable	Category	No. of examined milk samples (No. of herd)	No. of positive sheep's milk samples (No. of herd)	No. of positive goat's milk samples (No. of herd)	Frequency of positive milk samples (%95 CI)	*p* value
Region	South	120 (6)	3 (2)	1 (1)	3. 33 (2.6.-7.98)	0.00068
	North	106 (5)	4 (2)	2 (1)	5.66 (3.01-10.41)	
	Center	134 (7)	14 (5)	7 (4)	15.67 (10.48-22.77)	
History of abortion	With history	160	15	8	14.38 (9.78-20.65)	0.00048
	Without history	200	6	2	4.00 (2.04-7.69)	
Season	Spring	99	5	1	6.06 (1.68-19.61)	0.000022
	Summer	88	1	1	2.22 (0.87-5.57)	
	Autumn	87	9	7	18.18 (12.38-25.71)	
	Winter	86	6	1	8.13 (2.79-3.01)	
Age	< 2 years	109	2	1	2.75 (0.94-7.78)	0.015
	2 – 4 years	222	15	8	10.36 (7.00-15.07)	
	> 4 years	29	4	1	17.24 (7.60-34.55)	
Species	Sheep	180	21	-	11.66	0.038
	Goat	180	-	10	5.55	
Total		**360 (18)**	**31 (9)**		**% 8.611 (%50)**	

Gel Extraction:

To identify *C. abortus*, PCR products containing 343-bp gene fragments were sent to (Pishgam Biotechnology Co.) for sequencing after gel purification (Expin Combo GP-mini, Co Gene All, South Korea). (Figure 2).

Phylogenetic inferences

The phylogenetic tree was constructed based on the neighbor-joining analysis of helicase partial gene. This analysis revealed that two isolates of *C. abortus* were closely clustered with other *C. abortus* isolates from GenBank with more than 99.0% similarity (Figure 3).

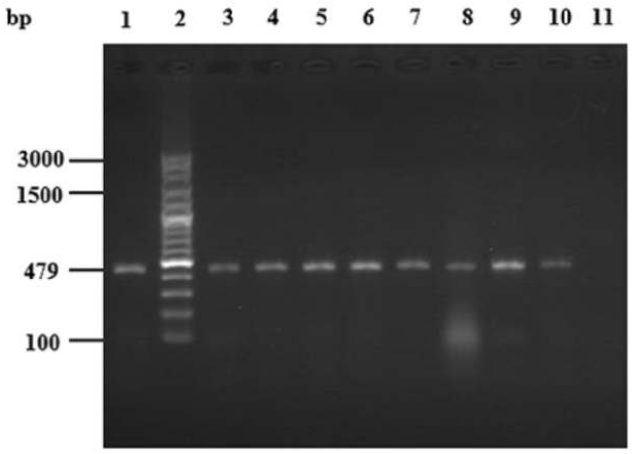

Figure 1.
Agarose gel image of an amplified fragment of the *C. abortus ompA* gene (479 bp) using PCR. Lane 1, Positive control; Lane M, 100 bp DNA size marker (SMOBIO Technology INC., Taiwan); Lanes 2, 3, 4, 5, 6, 7, 8, and 9, positive samples for *C. abortus*; Lane 10, negative control.

Discussion

Chlamydia is the cause of a variety of pathological syndromes in small ruminants. Among many, the most common clinical expression of infection is abortion that brings notable financial losses and risks to human health, particularly in pregnant women [3]. The present study was the first-ever epidemiological study on *C. abortus* in sheep and goats in West Azerbaijan province. Results of the present study revealed

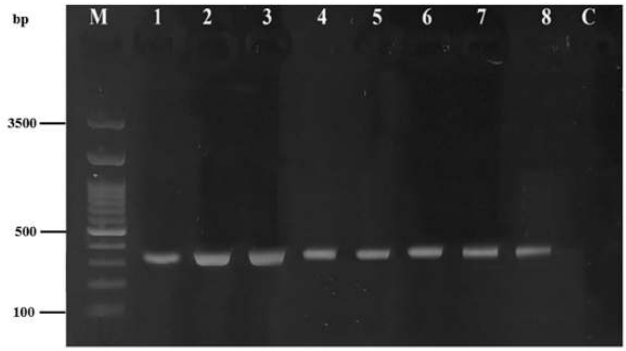

Figure 2.
PCR amplification of partial helicase gene from *C. abortus* bacteria. M: 100-bp DNA size marker; Positive samples: 1, 2, 3, 4, 5, 6, 7, and 8; C: Negative control

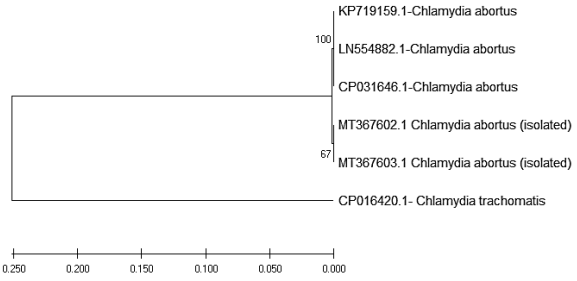

Figure 3.
Evolutionary analyses were conducted in MEGA X blast to show phylogenetic positioning of MT367602.1 and MT367603- *C. abortus* based on partial helicase gene, employing maximum likelihood available in GenBank sequences. Numbers on nodes indicate the bootstrap values.

that 8.1% of all examined raw milk samples were positive for *Chlamydia spp.* The prevalence of *C. abortus* in sheep and goats' raw milk has been reported by many researchers from Iran and other countries [24-26]. In a study in Mexico, the prevalence of *C. abortus* in goat milk was reported at 4.87% using the ELISA test [27]. Serological studies showed that 9% and 25% of sheep flocks of Khuzestan and Shahre-Kord provinces in Iran had antibodies against *C. abortus,* respectively [26, 28]. Pinheiro Junior et al. showed that 21.5% of sheep in Alagoas-Brasilia had antibodies against *C. abortus* and 77.7% of the population had at least one seropositive animal [29]. Huang et al. reported that 20.9% of Tibetan sheep had chlamydial antibodies [30].

The results of the current study showed that the prevalence of the *C. abortus* in sheep's milk (11.67%) was significantly more than goats' milk (5.56%). A study in Mexico showed that the prevalence of *C. abortus* in sheep and goats was 22.6% and 4.9% respectively [27]. The prevalence of *C. abortus* in sheep and goats with an abortion history was 14.38% which was significantly higher than of those without abortion history [4%]. This finding is consistent with other studies reported from Iran and other countries [24, 28]. The reason for this finding is that protective immunity does not develop when non-pregnant sheep are infected, and it can result in abortion [31, 32].

In a study in Iran, the prevalence of *C. abortus* in milk and other samples conducted by the molecular method in Tehran, Lorestan, Qom, Fars, Bushehr, East Azerbaijan, Khuzestan, and Chaharmahal va Bakhtiari provinces and results were 37.7%, 32.9%, 30.3%, 30.3%, 19.6%, 17.5%, 15.6%, and 52%, respectively [26]. The last reported finding is in accordance with those of Zaibet et al. [33] who reported that the risk of chlamydial infection in sheep is multiplied by 4 and 1.08 in the presence of cattle and goats on the same farm. We also found that flocks exposed to Chlamydiae showed not only risks of abortions, but also high sheep mortality rates. Indeed, chlamydial infection induces high animal mortality that finally reduces the financial capital of breeders and increases the costs of production [34]. Although the proportion of the positive sample in female sheep and goats was higher than male animals, sex was not significantly associated with the chance of seropositivity. Also, the seroprevalence of antibodies against *C. abortus* was not statistically different among age categories. Similar results were reported by McCauley et al. [35] who studied on seroprevalence of *C. abortus* in Australian sheep and Cubero-Pablo et al. [36] who reported seroepidemiology of chlamydial infection of wild ruminants in Spain. In a study in Turkey, the prevalence of *C. abortus* in ovine was 2.1% [37]. In other countries, the prevalence of chlamydia in milk was reported in the range of 3.70–61.0% so that the maximum and minimum prevalence of chlamydia in milk belonged to Sweden and Germany, respectively [27, 38-43].

It was shown that the geographical location may be a risk factor for *C. abortus* infection in sheep and goats. In the present study, the results showed that in the central region, the frequency of positive milk samples for *C. abortus* was 15.67%, nearly five times higher than in the south region (3.33%). This finding indicates that the sheep and goats populations in the south region are at lower risk of infection. The highest prevalence of *C. abortus* was in the region surrounded by mountains and Plateau [24, 26, 28]. The reasons for these differences in the prevalence of *C. abortus* might be the high density of livestock population in the central region than south of West Azerbaijan province.

The results of the present study showed that animals in the age group >4-year-old had the highest

prevalence of *C. abortus* (17.24%), which is in agreement with other studies [2, 44-46]. Qin et al. reported a significant correlation between age and infection, a by the increase of the age, the seroprevalence of *C. abortus* infection went up all the time, indicating that there may be a cumulative likelihood for exposure to *C. abortus* infection with age [47]. In a study by Esmaeili et al., the prevalence of *C. abortus* infection in small ruminant flocks according to age was 23.91% in the age group of 5-6 years [45].

The seasonal prevalence of *C. abortus* infection ranged from 2.22% to 18.11%. The highest prevalence (18.11%) was in autumn, and the lowest was in summer (2.22%). This finding of the current study was in agreement with similar studies from Iran and other countries [48]. In a study by Shi-Feng Hu et al. [48] in a seasonal survey of the *C. abortus*, the higher prevalence was in the autumn season.

In the present study phylogenetic analysis based on helicase gene revealed that the two sequenced isolates were almost identical with more than 99% similarity with the other *C. abortus* isolates from GenBank. In a study by Seth-Smith et al., phylogenetic analysis of a total of 64 genomes shows a deep structural division within the *C. abortus* species with a major clade displaying limited diversity. Also, the number of variable nucleotide positions across the sampled isolates is significantly lower than those published for *C. trachomatis* and *C. psittaci* [49]. Finally, regarding the public health issue of chlamydiosis, we suggest serological and molecular surveys on other species of livestock in West Azerbaijan province and other provinces of Iran will be necessary to clarify the picture of *C. abortus* infection in the country.

The prevalence of *C. abortus* infection in sheep and goats' milk was determined for the first time in West Azerbaijan, Iran. It was concluded that sheep and goats can play an important role in the epidemiology of Chlamydiosis as the reservoir for *C. abortus*. Our study showed that genetic diversity appears to be very stable. The molecular detection of *C. abortus* using the nested-PCR method in milk samples showed that PCR can be used as an easy and reliable approach for detecting *C. abortus*. The prevalence of *C. abortus* was higher in sheep's milk than in goat. Therefore, the consumption of sheep milk exposes humans to a higher risk of Chlamydial infection.

Materials & Methods

Study areas

West Azerbaijan province is in the northwest of Iran with over 3.5 m population. Urmia city is the center of the province. This province has diverse climates and geographical areas (e.g. relatively flat terrains, mountains, and the coasts of Urmia Lake). The climate is mostly featured with rainy winds of the Mediterranean and the Atlantic Ocean (https://www.britannica.com/place/Azerbaijan-region-Iran) (Figure 4). This province is very important in agricultural and animal production in Iran.

Milk sampling

A total number of 360 milk samples were randomly collected from sheep (n=180) and goats (n=180) belong to 36 different flocks in West Azerbaijan Province. The flocks were selected from three different geographical regions including the north, center, and south of the province. A total of 160 milk samples were from flocks with abortion history, and the other 200 samples were taken from flocks without abortion history throughout the year 2018. The sampled animals were classified into three age groups (<2 years old, 2-4 years old, and >4 years old). We avoided sampling from pregnant, early lactating dairy animals (<100 DIM) because the metabolic stress of early lactation may lead to immunosup-

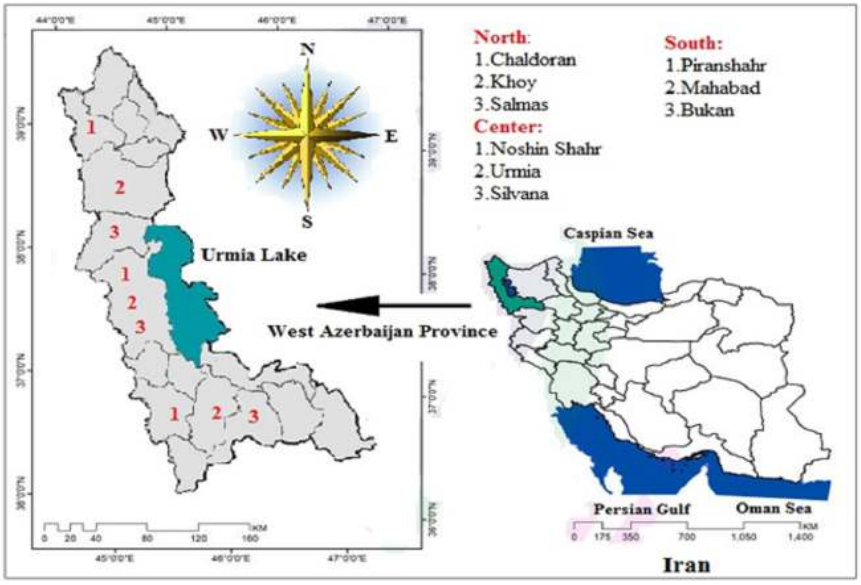

Figure 4.
The schematic map of the study areas, West Azerbaijan, Iran.

pression and therefore, an increase in disease susceptibility [20]. There has been no attempt to determine the cause of abortion in flocks with abortion. The milk samples were placed next to ice and transferred to the microbiology laboratory of the faculty of veterinary medicine.

DNA Extraction

Initially, the samples processed following a protocol described by White et al. [21]. Genomic DNA Extraction Kit (Favorgen, Taiwan) was used to extract DNA from milk samples according to kit's manufacturer instructions. The extracted DNA was quantified using NanoDrop 2000c (Thermo Scientific, USA) and kept at -20˚C until later use in PCR.

Amplification of 16s rRNA gene using Nested-PCR

Nested-PCR targeting the *16S rRNA* gene was used for molecular detection of *Chlamydia spp.* Using primers described by Messmer et al. [21] and modified by Longbottom et al. [8, 22] (Table 2). The first stage of the nested-PCR carried out by using Taq DNA Polymerase Master Mix RED (Amplicon, Denmark). The PCR reaction was prepared in 25 µl volume consisting of 5 µl of DNA template, 50 picomole of each primer (16SIGF, 16SIGR), 12.5µl of the master mix, and 6.5 µl of distilled water. For the second stage, PCR reaction was prepared as described above, except for the DNA template, for which 2.5 µl of 1:100 diluted PCR product from the first stage was used. The thermal cycling condition was described according to Messmer et al. [21]. The PCR products were electrophoresed on a 1.5% and 2% agarose gel stained with safe stain (Quanta. England) for stages 1 and 2, respectively. The gels were visualized through Ingenius Gel Documentation (Syngene Bio-Imaging, UK) (Figure 5).

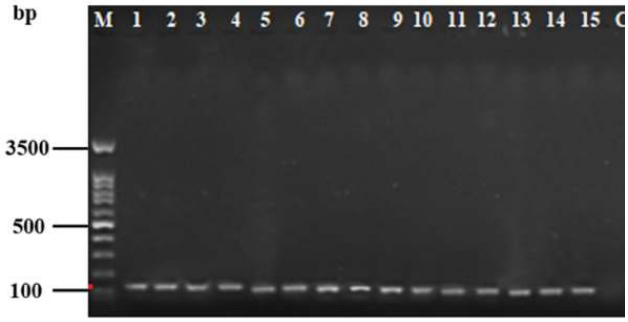

Figure 5.
Agarose gel image of an amplified fragment of the *C. abortus 16S rRNA* gene (127 bp) using nested-PCR. Lane 1, Positive control; Lane M, 100-bp DNA size marker (SMOBIO Technology Inc., Taiwan); Lanes 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15, positive samples for *C. abortus*; Lane C, negative control.

Amplification of the ompA gene in C. abortus

For the identification of *C. abortus ompA* gene was amplified using primers previously described by Creelan et al. (Table 2). The PCR reaction was performed in 25 µl volume comprising 5 µl of extracted DNA, 50 pmol of each primer (ompA 1, ompA 2), and 12.5µl of master mix. Amplification conditions were described according to Creelan et al. Amplified products were electrophoresed on 1% (w/v) agarose gel containing safe stain and then visualized using Ingenius Gel Documentation (Syngene Bio-Imaging, UK) (Figure 1).

Table 2.
The list of primers used for detection of *C. abortus* based on 16S *rRNA*, *ompA* and helicase genes.

Application	Primer Name	Sequence (5' to 3')	Amplicon length (bp)	PCR step (°C/seconds)				cycles
				pre denaturation	Denaturation	Annealing	Extension	
PCR	16SIGF	ACGGAATAATGACTTCGG	436	95/180	94/30	70/30	72/45	45
	16SIGR	TACCTGGTACGCTCAATT						
Nested PCR	F	ATAATGACTTCGGTTGTTATT	127	95/120	94/60	55/30	72/60	35
	R	TGTTTTAGATGCCTAAACAT						
PCR	OMPA-1	TGGTATTCTTGCCGATGAC	479	95/180	94/30	70/30	72/45	45
	OMPA-2	GATCGTAACTGCTTAATAAACCG						
PCR	12SM-FW	CTAGAGGAGCCTGTTCTATAATCGATAA	343	93/120	93/30	63/30	72/45	40
	12SBT-REV2	AAATAGGGTTAGATGCACTGAATCCAT						

Amplification of the Helicase gene in C. abortus

In order to amplify the helicase gene was used the PCR primers and conditions described by Cantekin, et al. [23] (Table 2).

Nucleotide sequencing

The PCR products of the helicase gene of *C. abortus* isolates were sent to SinaClon Company (Tehran, Iran) for sequencing. The obtained nucleotide sequences of the helicase gene were searched against GenBank (National Centre for Biotechnology Information, Rockville Pike, and Bethesda, USA) using the advanced BLAST similarity search option and compared to the helicase sequences of *Chlamydia spp.* from GenBank. Nucleotide sequences were aligned and compared to other nucleotide sequences from GenBank using Clustal-W and phylogenetic tree was generated using the neighbor-joining method in MEGA software (version X; Biodesign Institute, Tempe, USA).

Statistical analysis of data

The epidemiological data were analyzed using the *Chi*-square test in SPSS version 22 (IBM Corp. Armonk, NY, USA). Differences with a p value <0.05 were considered significant.

Authors' Contributions

FT and AO conceived and planned the experiments. FT, AO, and KM carried out the experiments. FT and AO contributed to sample preparation. AO and KM contributed to the interpretation of the results. All authors took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analyses, and the manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Vitamin E and hCG enhance the immunomodulatory properties of LPS-induced mesenchymal stem/stromal cells

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ABSTRACT

Mesenchymal Stem/Stromal Cells (MSCs) have been applied to modulate various immune-mediated conditions. Prolonged culture of MSCs in vitro reduces their therapeutic efficacy. Pretreatment of the cells with some chemical agents during in vitro expansion could overcome this limitation. This study intended to determine whether pretreatment of adipose-derived MSCs (ASCs) with Human chorionic gonadotrophin (hCG), a glycoprotein hormone, and Vitamin E, an antioxidant, will improve their immunomodulatory ability. In this regard, ASCs were harvested from human processed lipoaspirate. LPS-induced ASCs were preconditioned with 1 mg of hCG and 600 µM of vitamin E for 24h. TSG-6, COX-2, IL-1β, and IL-6 were assessed at the mRNA level in preconditioned and control groups. ASCs were also co-cultured with peripheral blood mononuclear cells (PBMCs) in vitro to determine the functionality of these cells. Results showed that hCG and vitamin E significantly downregulate the pro-inflammatory COX-2, IL-1β, and IL-6 gene expression, while they did not significantly increase TSG-6 expression. Besides, the co-culturing of pretreated ASCs with PBMCs demonstrated that the amount of PBMCs in treated groups (with hCG and vitamin E) was significantly lower than in control groups. These findings revealed that the preconditioning of ASCs with hCG and vitamin E might enhance their immunoregulatory capacity.

Keywords

hCG, Immune regulation, ASCs, pretreatment, stem cells, Vitamin E

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Abbreviations

hCG : human Chorionic Gonadotropin
LPS: Lipopolysaccharide
MSCs : Mesenchymal Stem/Stromal Cells
ASCs : Adipose-derived Mesenchymal Stem/Stromal Cells
PBMCs : Peripheral Blood Mononuclear Cells
TSG-6 : Tumor necrosis factor-inducible gene 6COX-2 :Cyclooxygenase-2
IL-1β : Interleukin 1 beta
IL6 : Interleukin 6
Tregs : Regulatory T cells
TLRs : Toll-Like Receptors
poly(I: C): PolyInosinic:polyCytidylic acid
LH : Luteinizing Hormone

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Introduction

Mesenchymal stem/stromal cells (MSCs), capable of self-renewal and multi-lineage differentiation, initially attracted biomedical scientists considering their reparative properties to replace damaged cells [1]. Later, the "stromalness" concept attributed most MSCs' therapeutic outcomes to the paracrine and trophic signals [2]. Recently, the role of MSCs as guardians against excessive inflammatory responses describes many of the observed favorable effects [1, 3]. Further, it became apparent that the immunomodulatory functions of MSCs are licensed by their surrounding inflammatory environment [3]. Among all human tissues which could be regarded as sources of MSCs, adipose tissue became an attractive one that was mainly due to less invasive harvesting methods, high proliferation properties, and considerable stromal function [2, 4].

To acquire sufficient cells for administration, they should be cultured for a long time, which may change their properties and make them unsuitable for clinical applications [5]. On the other hand, after systematic administration, inflammation conditions or harsh *in vivo* environments may impair cell therapeutic response, viability, homing, and biological potentials [6, 7]. The physiological features of Adipose-derived Mesenchymal Stem/Stromal Cells (ASCs) should be restored to enhance their survival rate and resolve these problems [8].

Diverse methods are developed to improve the functional properties of MSCs, such as preconditioning of the cells with bioactive molecules, genetic manipulation, and modification of the culture condition [8, 9]. In several previous studies, investigators explored the preconditioning effects of different agents on MSCs [10-13]. Moreover, the immunomodulatory ability of Naïve MSCs is low in unstimulated conditions; thus, MSCs need to be activated with proinflammatory stimuli like IFN-γ or lipopolysaccharide (LPS), or an immunosuppressive Toll-like receptor 3 (TLR3) agonist polyinosinic-polycytidylic acid (poly(I: C)), to gain their full biological potential [14-16].

For about a century, α-tocopherol (vitamin E) has been known as a potent antioxidant and a fat-soluble vitamin [17, 18]. This vitamin could trap oxygen and nitrogen radicals and protect the cell membrane by protecting polyunsaturated fatty acids (PUFAs) (19). Azzi first explained the non-antioxidant properties of this vitamin [20]. Many studies revealed that it could also modulate cell signaling and gene expression [21]. The immunomodulatory potential of vitamin E also has been reported *in vivo* [22, 23].

Accumulated evidence indicated a close relationship between inflammation and oxidative stress, one of which can promote another. Reactive species can raise proinflammatory gene expression by arousing intracellular signaling cascade. On the other hand, inflammatory cells could generate more reactive oxygen species (ROS), resulting in augmented oxidative stress at the site of inflammation. Treatment with antioxidants is thought to be a desirable approach to prevent inflammatory diseases caused by oxidative stress [24].

As the most abundant and essential free radical scavenger, Vitamin E plays its antioxidant role by decreasing overall oxidative stress. The protective role of vitamin E therapy in inflammatory diseases like atherosclerosis and diabetes mellites was established by Elbeltagy and Alshiek et al. [25, 26].

Human chorionic gonadotrophin (hCG) is a glycoprotein hormone that plays a significant role in maintaining a pregnancy [27]. Besides organs of the reproductive system, receptors for hCG shared with luteinizing hormone (LH) were represented on T and B lymphocytes and macrophages (28). The binding sites of hCG on non-reproductive tissues indicate additional roles, such as immune modulation [29]. This hormone can regulate T cells, dendritic cells, and natural killer cells and enhance vascularization [30-32]. Fuchs first evidenced the immunosuppressive effects of hCG in 1980 [33]. It was also used as a therapeutic agent in autoimmune diseases, e.g., rheumatoid arthritis [34]. The effect of hCG on stem cells may be attributed to its receptor on MSCs as they have been previously described [35].

In addition to the integral hCG molecule, linear tetrapeptide originating from the β-hCG, AQGV, exert a potential novel immune-modulatory effect [36]. This synthetic oligopeptide, currently developed under the product name EA-230 has been shown to inhibit the inflammation, disease severity, and mortality in high-dose LPS-induced inflammatory response [37]. Pramanik found that Nuclear factor-kappa B (NF-κB), the master regulator of many proinflammatory genes, was downregulated after treatment with hCG [38]. Therefore, in line with Bai results, hCG downregulated the mRNA expression of IL-1β and IL-6 and could inhibit the production of pro-inflammatory cytokines, which can help reduce inflammatory symptoms and Improvement of autoimmune diseases [39].

Abbreviations-Cont'd

ROS : Reactive Oxygen Species
NF-κB: Nuclear Factor kappa B
PBS = Phosphate-Buffered Saline
PUFAs = PolyUnsaturated Fatty Acids
DMEM = Dulbecco's Modified Eagle's Medium
RT-qPCR = quantitative Real-Time Polymerase Chain Reaction
XTT = XML Tunneling Technology.

The current study aimed to determine whether pretreatment of human ASCs with hCG and vitamin E can decrease the pro-inflammatory status of MSCs caused by LPS and enhance their immunomodulatory potential.

Results

Characterization of ASCs

Isolated cells from adipose tissues were successfully cultured and passaged *in vitro*, and they exhibited spindle-shaped morphology, as shown in Figure 1A. Osteogenic and adipogenic differentiation of the cells was determined by the appearance of calcium deposits and lipid droplets, respectively (Figures 1B, 1C, and 1D).

In addition, ASCs were characterized by positive expression of CD90 (98.7%), CD44 (99.3%), CD73 (98.9%), CD13 (99.0%) and negative expression of CD14 (1.21%), HLA-DR (1.08%), CD45 (1.11%) and CD34 (1.71%), and surface markers (Figure 1E).

Cell viability

Effects of hCG and vitamin E on ASCs viability were investigated using our established protocol, which demonstrated that preconditioning of the cells with these substances does not exert remarkable toxicity against ASCs (40, 41).

Increased secretion of proinflammatory cytokines in LPS-primed ASCs

Human ASCs were incubated with 5µg/ml of LPS for 4 hours. First, we observed the morphology of LPS-primed ASCs, which were spindle-shaped (Figure 2A).

The expression of genes related to the inflammation in LPS-primed ASCs was determined by qRT-PCR, as presented in Figure 2B. The results demonstrated that LPS increased mRNA expression of Cox-2, TSG-6, IL-1β, and IL-6 compared to unstimulated ASCs as the negative control (Figure 2B). These data confirm that LPS leads to the induction of inflammatory response in ASCs.

Treatment of LPS-primed ASCs with hCG and vitamin E

To evaluate the effects of hCG and vitamin E, we incubated LPS-primed ASCs with 10 IU hCG for 48 h and 600 µM vitamin E for 48 h. The morphology of ASCs after LPS-priming and pretreatment with hCG and vitamin E was similar to the control group and displayed a fibroblastic-like appearance (Figure 3A and 3B).

The mRNA expression of inflammatory cytokines in pretreated LPS-primed ASCs was determined by RT-qPCR. The overexpression of TSG-6, Cox-2, IL-1β, and IL-6 in LPS-primed ASCs was downregulated by pretreating cells with 10 IU hCG (Figure 3C).

Besides, vitamin E treatment of LPS-primed ASCs significantly reduced the expression level of Cox-2, TSG-6, IL-1β, and IL-6 (Figure 3D). These data demonstrated that after treatment with hCG and vitamin E, the increase of inflammatory cytokines in LPS-primed ASCs was significantly reversed so that these treatments could increase the immunomodulatory properties of LPS-primed ASCs compared to the control group.

The effect of pretreated ASCs on inhibition of PBMCs proliferation

We evaluated the proliferation of PBMCs co-cultured with treated ASCs using XTT colorimetric assay. We considered activated PBMCs proliferation as 100%, and the proliferation inhibition percentage of co-cultures was calculated using the below equation. The significance of differences among data was examined at the confidence level of 95% ($p < 0.05$) using the *t*-test. All experiments were performed in triplicate. Proliferation ratio: (Co-culture-OD / PBMCs-OD) × 100. Results demonstrated that the proliferation of activated PBMCs (stimulated with PHA) was inhibited when co-cultured with ASCs (Figure 4A). The proliferation retardation percentage is 25.2% that is statistically significant ($p < 0.05$). However, hCG-treated ASCs could reduce the proliferation ratio of PBMCs more than untreated ASCs, down to 64.09%, and that is highly significant ($p < 0.01$) (Figure 4A). Also, Ethanol treated ASCs as the control group of vitamin E pretreatment, could significantly reduce the proliferation of PBMCs (24.94%), and the inhibition activity of vitamin E treated ASCs was stronger than its control group with a retardation percentage of 50.6% and is statistically significant ($p < 0.001$) (Figure 4B).

Differences in proliferation in hCG and vitamin E treated ASCs under the co-culture conditions were significant compared to their control groups untreated and ethanol-treated ASCs, respectively ($p<0.05$) (Figure 4A (a) and 4B (b)).

Discussion

MSCs appear to be a decent choice for cellular therapy of immune-mediated disorders [42]. Over prolonged preparation passages, MSCs may lose their therapeutic efficiency [43]. Studies have shown that various surrounding microenvironments affect MSCs' paracrine signaling and polarize them to proinflammatory or anti-inflammatory phenotypes [44, 45]. Pretreatment of the cells with some chemical

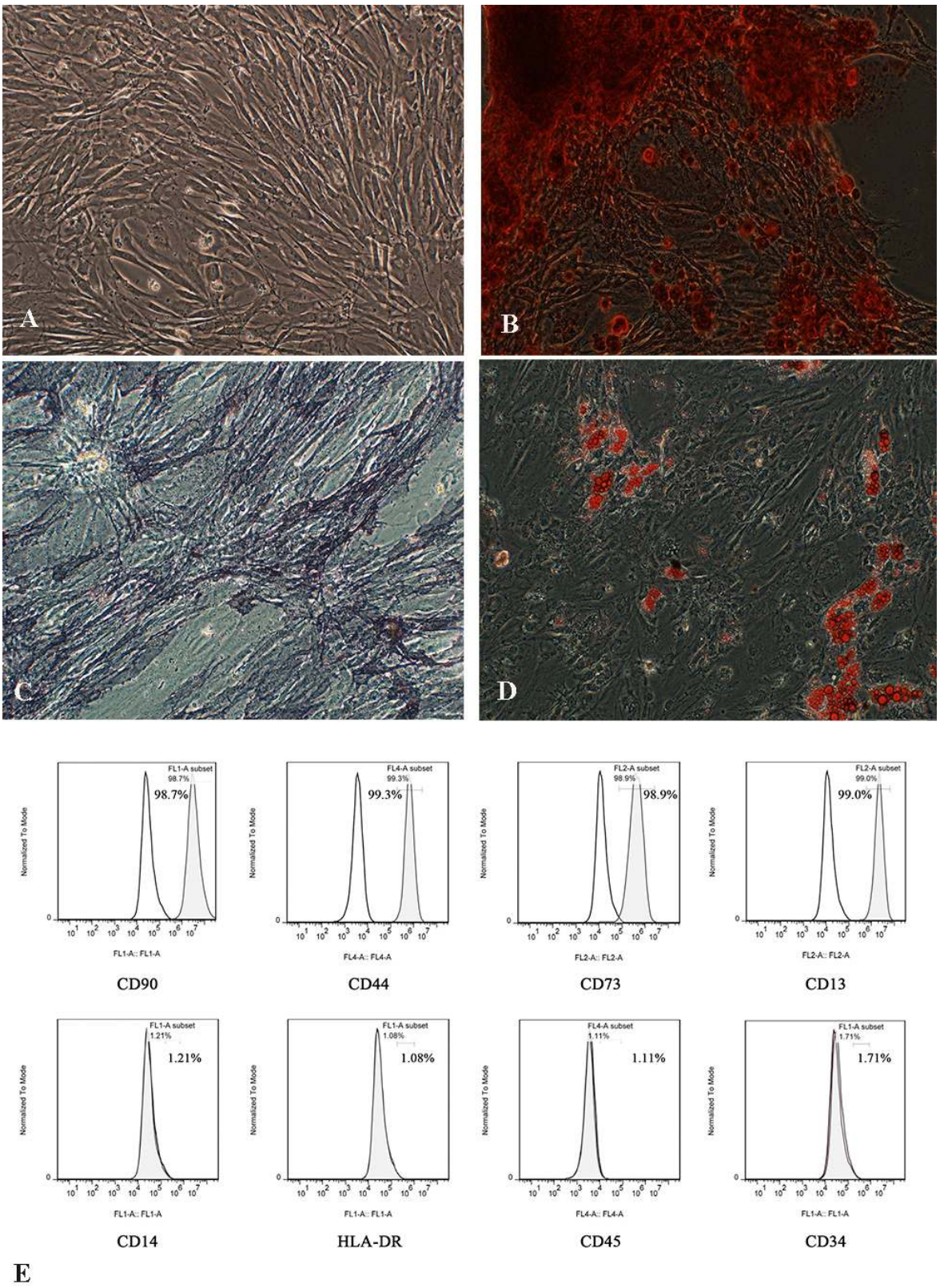

Figure 1.
Characterization of human Ad-MSCs (A) undifferentiated human Ad-MSCs represent spindle-shaped morphology (Scale bar = 200 µm). (B) Osteogenic differentiated human Ad-MSCs were stained with alizarin red (Scale bar = 100µm). (C) Alkaline phosphatase assay was used to confirm osteogenic differentiation (Scale bar = 200µm). (D) Adipogenic differentiated human Ad-MSCs were stained with oil red O (Scale bar = 100µm). (E) Flow cytometric analysis showed that almost all cultured Ad-MSCs expressed CD90 (98.7%), CD44 (99.3%), CD73 (98.9%), CD13 (99.0%), whereas a small portion of the cells expressed CD14 (1.21%), HLA-DR (1.08%), CD45 (1.11%) and CD34 (1.71%). Expressions of cell surface markers of Ad-MSCs are shown as compared with their respected isotype controls.

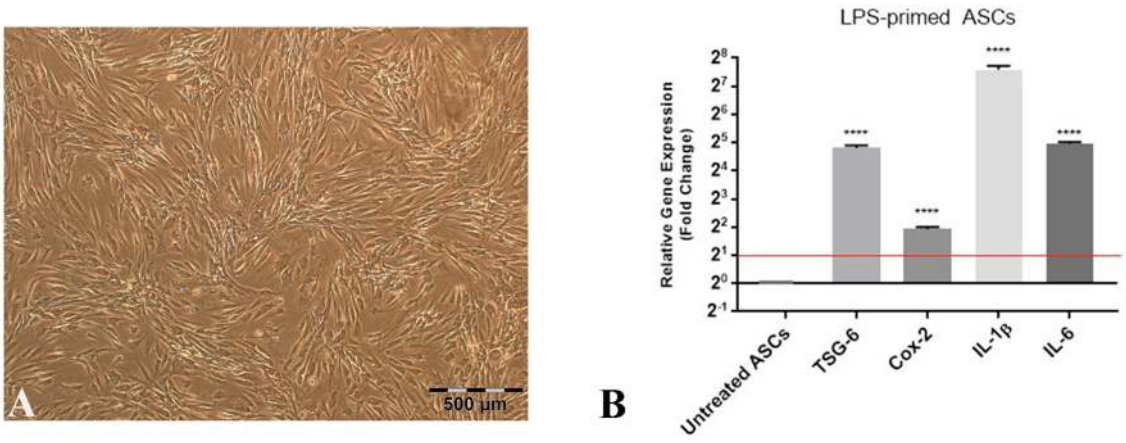

Figure 2.
Effect of LPS-primed Ad-MSCs on expressions of genes involved in inflammation. (A) Spindle-shaped morphology of LPS-primed Ad-MSCs (Scale bar = 500µm). (B) mRNA quantification of cytokines in LPS-primed Ad-MSCs: results showed that LPS pretreatment increases the inflammatory properties of Ad-MSCs. Data are illustrated as mean ± SEM (n=3) and presented as fold change (log 2) of expressions in preconditioned versus untreated cells. Four stars represent *p*-value < 0.0001.

agents during *in vitro* expansion helps overcome these limitations by restoring the physiological activities and enhancing their biological potency [46]. It is estimated that pretreatment of MSCs isolated from different sources might result in variable responses [47]. Here, we used LPS-primed ASCs. Our observations demonstrated that the licensing of ASCs by LPS and their pretreatment with hCG and vitamin E could significantly alter immunoregulatory genes' expression and enhance these cells' anti-inflammatory potential in vitro.

Toll-like receptors (TLRs) perceive danger alarms from various pathologies. Their activation recruits immune cells to the site of injury. Waterman observed that MSCs could be recruited likewise. MSCs express TLR3 and TLR4. They discovered that the engagement of some TLR-agonists could intensely regulate their migration and secretion of immune-modulating agents. They also identified that TLR4-primed MSCs, generally exert proinflammatory mediators, while TLR3-primed MSCs express mostly immunosuppressive ones [48]. It suggested that stimulation of TLR4 with Lipopolysaccharide (LPS) could mimic a proinflammatory milieu [49, 50]. In this investigation, we employed LPS-priming to provide a proinflammatory signature of ASCs. In agreement with our observations, LPS was shown to affect ASCs by overexpression of inflammatory cytokines, namely, TSG-6, COX-2, IL-1β, and IL-6 [51].

TSG-6 has been identified as a critical mediator of the anti-inflammatory effects of human MSCs. TSG-6 constricts inflammatory responses by inhibiting neutrophils' invasion into the inflammatory sites [52]. Roddy determined that the siRNA knockdown of TSG-6 impeded these cells' anti-inflammatory functions on damaged corneal epithelial cells [53].

COX-2 is a pivotal enzyme in prostaglandin E2 synthesis, a well-known participant in various autoimmune diseases like rheumatoid arthritis [54, 55], and COX inhibitors are considered therapeutic targets for inflammation-mediated disorders [56, 57]. IL-6 is considered a pleiotropic proinflammatory cytokine involved in various physiological functions, including inflammation via transcription (STAT) activators, signal transducers, and NF-κB signaling pathways. There is evidence that blockade of IL-6 prevents the progression of autoimmune diseases and tumor formation [54, 55]. IL-1β is also a pro-inflammatory cytokine regulated through the NF-κB pathway [58]. These two partners are involved in many inflammatory conditions, and they are considered therapeutic targets for inhibitory agents [59, 60]. Our data showed that pretreated LPS-primed ASCs with hCG and vitamin E exert anti-inflammatory and immunomodulatory activities. In particular, LPS-primed ASCs preconditioned with hCG, and vitamin E reduced the expression of proinflammatory COX-2, IL-1β, and IL-6 genes and maintained anti-inflammatory expression TSG-6 gene.

Our study also demonstrated that the co-culturing of pretreated ASCs with PBMCs significantly reduced proinflammatory and anti-inflammatory Gene expressions in treated groups (with hCG and vitamin E) compared with the untreated groups. Bofeng Li, in 2018, found that IL-6 has a substantial role in T cell expansion and promotes T cell proliferation [61]. It could be suggested that the amount of T cells reduced as a result of IL-6 reduction.

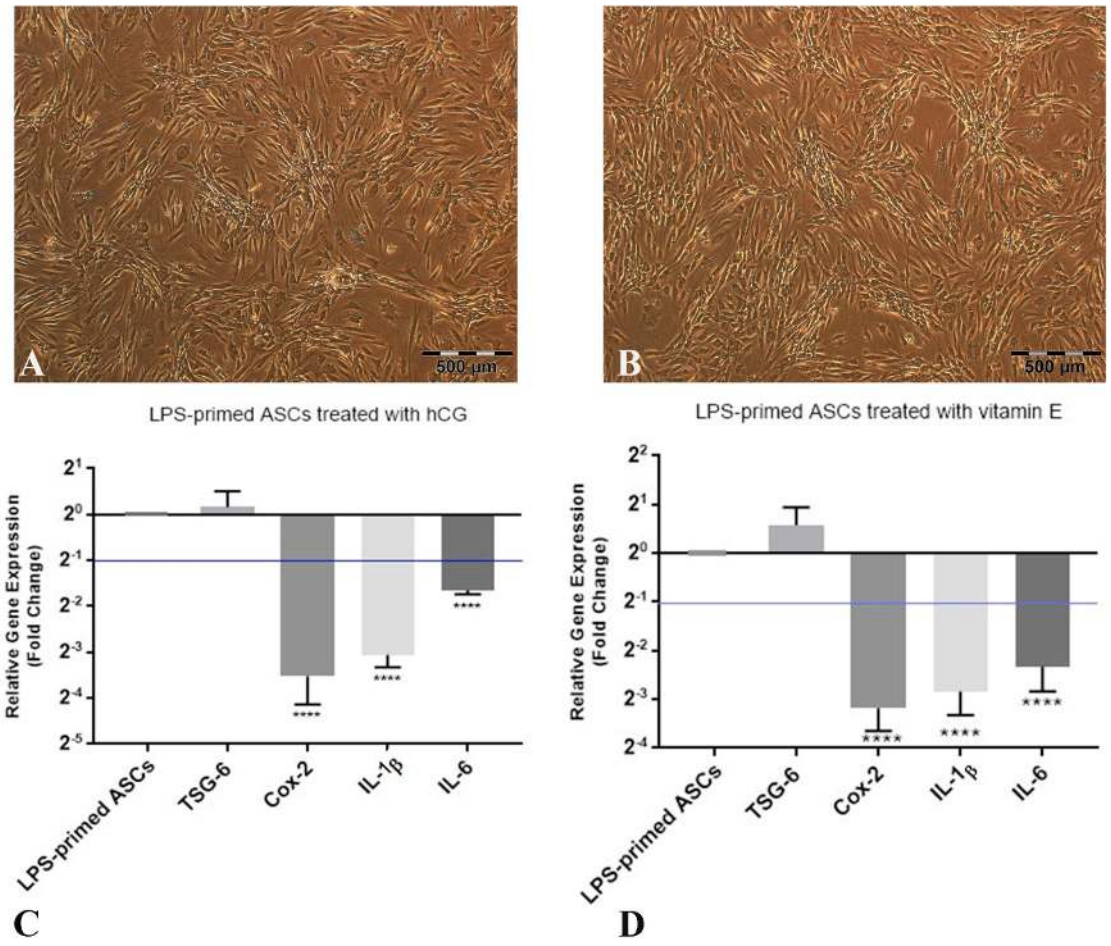

Figure 3.
Effect of hCG and vitamin E on the expression of genes involved in inflammatory .... in LPS-primed Ad-MSCs. (A and B) Spindle-shaped morphology of hCG and vitamin E treated LPS-primed Ad-MSCs, respectively (Scale bar = 500µm). (C) Treatment of LPS-primed Ad-MSCs with hCG decreases the mRNA levels of the inflammatory-related genes. (D) Vitamin E treatment reduces the expression of inflammatory cytokines in LPS-primed Ad-MSCs. Data are shown as mean ± SEM (n=3) and presented as fold change (log 2) of expressions in preconditioned versus untreated cells. Four stars representa *p*-value < 0.0001.

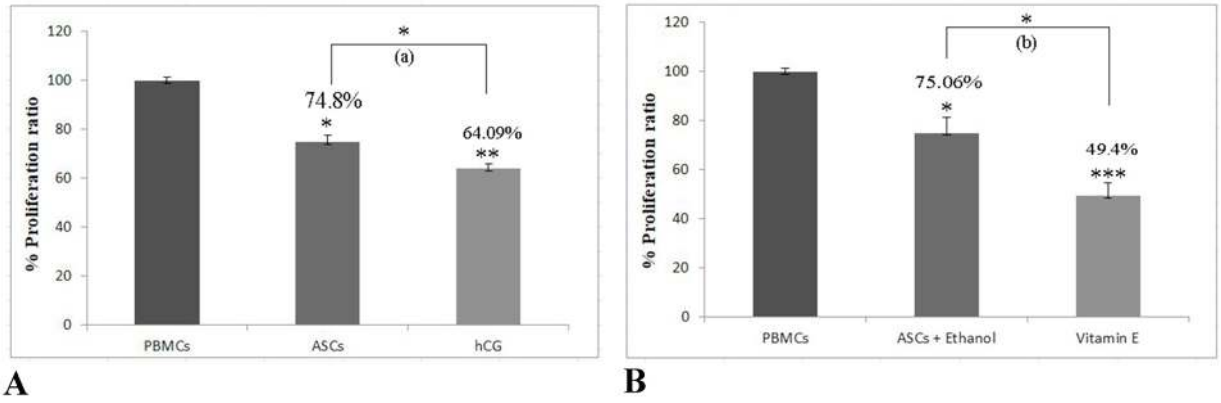

Fig 4.
Effects of treated Ad-MSCs on PBMCs proliferation. PBMCs were co-cultured with treated and untreated Ad-MSCs, and their proliferation was assessed using XTT. (A) PBMCs numbers were significantly reduced when they were co-cultured with untreated Ad-MSC (25.2%) and hCG (35.91%), and (B) Ethanol treated Ad-MSCs (24.94%), and vitamin E treated Ad-MSCs (50.6%) compared to the control group (PHA-PBMCs). Error bars display the mean ± SD, n=3, (*p < 0.05, **p < 0.01, ***p < 0.001).

In conclusion, hCG and vitamin E preconditioning were implied to improve the anti-inflammatory and immunomodulatory capacities of LPS-primed ASCs. It could be at least in part through inhibiting the expression of proinflammatory cytokines. Hence, it could be regarded as a potential therapeutic strategy.

Materials & Methods

ASCs isolation and culture

Liposuction aspirated wastes from subcutaneous adipose tissues were obtained from three healthy individuals undergoing liposuction surgery after getting informed consent at a cosmetic day clinic in Mashhad, Iran. The ACECR-Khorasan Razavi Biomedical Research Ethics Committee has approved this research (Code: IR.ACECR.JDM.REC.1398.008-009).

The aliquots of fat (200 ml) were washed 3-4 times with equal volume phosphate-buffered saline (PBS) supplemented with 0.1% penicillin-streptomycin (pen-strep) (Biosera, France), and then incubated for 1 hour at 37°C with freshly prepared 0.1% collagenase type I (Invitrogen, USA). They were shaken robustly and repeatedly for 5-10 seconds. Fetal bovine serum 10% (FBS; Gibco, USA) was applied for collagenase I inactivation. To remove adipose cell debris, we centrifuged them at room temperature (600 × g, 10 min).

Following washing the pellet with PBS and centrifugation (400 × g, 6 min), the collected cells were seeded in tissue culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM, Biowest, France) supplemented by 10% FBS and 0.1% pen-strep, and kept in a 5% CO2 incubator at 37°C. ASCs were purified based on their plastic-adherent capacity, and the medium exchange was performed twice a week. Cells were trypsinized (0.025%, Gibco, USA) following reaching confluency of 85 to 90%. ASCs at the third passage were employed in all the following experiments.

Characterization of ASCs

The Flowcytometric approach was applied to identify mesenchymal lineage-specific surface markers (BD Accuri C6, USA). A suspension of 2×105 cells was stained with 2 µg/ml of PE-conjugated CD73, CD13, FITC-conjugated CD90, CD34, CD14, and HLA-DR, and APC-conjugated CD44, CD45 antibodies for 45 min (all from Cytognos, Spain). Data analysis was carried out via FlowJo (7.6.1) software.

The multi-lineage potential of human ASCs was confirmed by inducing their differentiation into adipogenic and osteogenic lineages using the appropriate culture conditions. Briefly, adipogenesis was induced through the culture of ASCs in the presence of DMEM supplemented by 10% FBS, 200 mM indomethacin, 1 mM dexamethasone, and 10 mM β-glycerophosphate (all from Sigma Aldrich, Germany). After 14 days, the cells were rinsed with PBS and fixed in 10% formalin solution. The differentiation induction level was evaluated by applying oil red O staining (Sigma Aldrich, Germany) to indicate intracellular lipid droplets.

Additionally, osteogenic differentiation of the cells was evaluated qualitatively based on the cytochemical analysis. To do so, ASCs were incubated with osteogenic inductive media, including 50 mM ascorbate-2- phosphate (Sigma Aldrich, Germany), 10 mM β-glycerophosphate, and 0.1 mM dexamethasone for 21 days. They were then fixed and stained with alizarin red (Sigma Aldrich, Germany) to confirm the extracellular matrix's presence of calcium mineralization, secreted by differentiated cells.

Priming ASCs with LPS

Typically, ASCs were grown to 60-70% confluence in the growth medium before the start of an experiment. Lipopolysaccharide (LPS) was added (5µg/ml) to a fresh growth medium and incubated with the cells for 4 hours (Gibco, USA). Afterward, cells were washed with PBS and used for the subsequent analysis.

Treatment of ASCs with hCG and vitamin E

LPS-primed ASCs were treated with hCG (1 mg, Homapharmed, Iran) and vitamin E (600 µM, Sigma, Germany) for 24h. Non-treated and exclusive LPS primed ASCs are used as controls. After treatment, cells and culture medium were collected for the experiment

Table 1.
Primer sequences used for RT-qPCR.

Target Gene	Sequence	Product size (bp)
RPLP0 (NM_053275.4)	F: TGGTCATCCAGCAGGTGTTCGA R: ACAGACACTGGCAACATTGCGG	119
TSG-6 (NM_007115.3)	F: GCTGCTGGATGGATGGCTAAG R: CTCCTTTGCGTGTGGGTTGTAG	156
COX-2 (NM_000963.3)	F: CCAGAGCAGGCAGATGAAATACC R: ACCAGAAGGGCAGGATACAGC	168
IL-1β (NM_000576.2)	F: CCTCTCTCACCTCTCCTACTCAC R: CTGCTACTTCTTGCCCCCTTTG	186
IL-6 (NM_000600.4)	F: ACTCACCTCTTCAGAACGAATTG R: GCAAGTCTCCTCATTGAATCCAG	196
IL-10 (NM_000572.2)	F: GAGATGCCTTCAGCAGAGTGAAGA R: AGGCTTGGCAACCCAGGTAAC	114

RNA extraction and quantitative PCR

Total RNA was extracted using Tripure reagent according to the protocol provided by the manufacturer (Roche, Germany).

The purity and concentration of RNA samples were detected using Nanodrop ND-1000 spectrophotometer (Bio-Tek, USA), and the integrity of RNA samples was analyzed by gel electrophoresis.

Total RNA samples were treated by one unit of DNase I (Thermo Fisher Scientific, USA) to avoid genomic DNA contamination. Afterward, 1µg of total RNA was used for first strand cDNA synthesis using PrimeScript RT reagent Kit (Cat. #RR037A, TaKaRa) according to manufacturer's protocol.

The RT-qPCR was carried out using the Bio-Rad CFX-96 system (Bio-Rad, USA). Each reaction mixture contained 2 µl cDNA (0.1 diluted), 10 µl SYBR Green PCR Master Mix (Takara, Japan), and 1 µl of 10 pmol/ml mixture of forward and reverse primers in a final volume of 20 µl. Experiments were performed in duplicates. The ribosomal protein lateral stalk subunit P (RPLP0) gene was used as an internal control to normalize the expression level of the target genes. Primers were designed by AlleleID 6 software and are shown in Table 1.

To confirm PCR efficiencies for each gene we used pooled cDNA in 10-fold dilution series and used the $2^{-\Delta\Delta Ct}$ method for gene expression when the calculated slope was about ~ -3.3 equals to E = 95-105%.

Co-culture and lymphocyte proliferation assay

The pretreated ASCs effect on peripheral blood mononuclear cells (PBMCs) proliferation was investigated in the co-culture model.

Human PBMCs were isolated from healthy donors' blood using Ficoll-Paque (Biowest, Canada) density gradient centrifugation. The cells were stimulated with 205 mg/mL phytohemagglutinin for mitogenic stimulation (PHA, Sigma, Germany).

The ASCs were plated at 4×10^4 cells in a 48-well plate and left to adhere overnight. The next day, 1 mg hCG and 600 µM vitamin E were added to several wells for 48 hours.

Then, 10^5 stimulated PBMCs were added to each well and co-cultured with ASCs for 72 hours in a ratio of 1:1 of RPMI-1640: DMEM medium supplemented with 10% FBS and 0.1% penicillin-streptomycin (Biowest, Canada). Well groups are shown in Table 2.

The inhibition of PBMCs proliferation was measured using the 2H-tetrazolium salt XTT colorimetric method according to the manufacturer's instruction (Santa Cruz, USA).

Statistical analysis

The GraphPad Prism statistical program (version 7) and two samples *t*-test was used for data analysis. The values are reported as the mean of at least three independent experiments ± SD. Events with *p* values less than 0.05 were considered significant.

Table 2.
Well groups of proliferation co-culture.

Control groups	Sample groups
PHA stimulated PBMCs	-
Untreated ASCs	hCG treated ASCs
Ethanol treated ASCs	Vitamin E treated ASCs

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Authors' Contributions

Conception: MF, HRB, and HH. Planning: HH, MF, NSM, MMM, and MKN. Carrying out: SSH, SM, HH, and MKN. Writing: SM, SSH, HRB, and HH.

Conflict of interest

The authors declare no competing interest.

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Antibacterial effect of *Satureja hortensis* and *Salvia officinalis* essential oils against major bovine mastitis bacteria

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ABSTRACT

Treatment of bacterial diseases such as bovine mastitis with antibiotics has problems such as antibiotic resistance and drug residue in animal products. Essential oil of medicinal plants have antibacterial activity and are suitable alternatives. This study examined the antimicrobial activity of *Salvia officinalis* (sage) and *Satureja hortensis* (savory) essential oils on major mastitis-causing bacteria, including *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Escherichia coli*. Chemical compositions of essential oils were determined by gas chromatography-mass spectrometry. Minimum inhibitory concentration and minimum bactericidal concentration of oils were determined with serial broth dilution method using autoclaved whole milk rather than synthetic broth. The effect of sub-minimum inhibitory concentrations of essential oils on the growth curve of tested bacteria in milk was obtained in 0, 1, 2, 4, 10, and 24 hours. Major compositions of sage and savory essential oils were carvacrol (61.01%), thymol (20.41%), 1R-α-pinene (7.88%), eucalyptol (32.45%), thymol (28.24%), and α-pinene (13.42%), respectively. The minimum inhibitory concentration and minimum bactericidal concentration ranged 1.25-2.5% and 2.5-5% for savory, and 0.625-1.25% and 1.25-2.5% for sage, respectively. Savory and sage significantly decreased the *S. aureus* and *S. agalactiae* population in 4, 10, and 24 h ($p < 0.05$) and *E. coli* population in 10 and 24 h ($p = 0.01$). The sage and savory essential oils had antibacterial effects against three tested bacteria, and sage had a stronger effect than savory because of stronger antibacterial components (carvacrol and thymol). Further *in vivo* tests are recommended to evaluate the efficiency of these essential oils on the treatment of bovine mastitis.

Keywords

Antibiotic, medicinal plants, sage, savory

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Abbreviations

MIC: minimum inhibitory concentration
MBC: minimum bactericidal concentration
TSA: tryptic soy agar
DMSO: dimethyl sulfoxide
GC/MS: gas chromatography/mass spectrometry
EO: essential oil

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Introduction

Bovine mastitis is the most common disease of the dairy industry worldwide that has very economic losses, including discarded milk, some ineffective treatments as well as concerns about animal welfare and public health [1]. Mastitis is commonly treated with intra-mammary infusion of antibiotics, but cure rates are usually poor and various for some pathogens of mastitis, such as the cure rates of 20-57% for *Staphylococcus aureus* mastitis. Moreover, the appearance of resistance to an antibiotic in bacteria is a potential consequence of antibiotic usage [2]. According to these problems and concerns, alternative approaches are needed to control bovine mastitis. Essential oils are volatile secondary metabolites of low molecular weight derived from plants. These oils have antibacterial properties, with no reports of resistance after prolonged exposure to bacteria, and no side effects on human health, which makes them a potential weapon against bacterial diseases [3].

Salvia officinalis (sage) is one of the oldest and still the most popular medicinal plant that can grow wildly or in cultivation. Besides many other therapeutical properties, *S. officinalis* has anti-inflammatory, antiseptic, and broad-spectrum antibacterial activity [4,5]. *Satureja hortensis L.* (summer savory; savory) is a main *Satureja* species that has some applications in medicine and nutrition. Savory has different pharmacological effects, including antispasmodic, antimicrobial, antidiarrheal, and sedative [6,7].

There is some data on the antibacterial effects of sage and savory using synthetic media. In this study, we tested the antimicrobial efficacy of *Salvia officinalis* and *Satureja hortensis* oils in inhibiting the growth of major bovine mastitis pathogens (*Staphylococcus aureus, Streptococcus uberis*, and *Escherichia coli*) in milk. There is a complex food matrix in milk that nutrients such as proteins and fat may preserve pathogens of mastitis or decrease the antimicrobial effect of molecules [1], thereby for the usage of an antimicrobial agent for intra-mammary infusion it is necessary to assess the antimicrobial properties of these plant oils in milk.

Results

Chemical compositions of the essential oils

Major components of the savory oil were eucalyptol (32.45%), carvacrol (28.24%), α-pinene (13.42%), and thymol (9.71%), and those of sage were carvacrol (61.01%), thymol (20.41%) and 1R-α-pinene (7.88%) (Tables 1, 2).

MIC and MBC

The MIC and MBC of sage and savory essential oils on bacteria are provided in Table 3. Antimicrobial activity was confirmed against all tested microorganisms. The MIC and MBC ranged 1.25-2.5% and 2.5-5% for savory, and 0.625-1.25% and 1.25-2.5% for sage, respectively. Although savory, sage, and savory + sage essential oils showed an antibacterial effect against the three bacteria, sage oil was the strongest. It can be seen from the data in Table 3 that MBC and MIC of sage had the lowest effect compared with the other two essential oils.

Bactericidal kinetics of the oils

Figures 1-3 provide the effect of savory and sage on the growth curve of bacteria in milk. The initial bacterial count in the control and treatment samples for three bacteria was approximately 5.0 $\log_{10}$ cfu/ml. The bacterial population increased after 24 h to 12 $\log_{10}$ cfu/ml for *E. coli* and *S. agalactiae* and 10 $\log_{10}$ cfu/ml for *S. aureus* in the control samples. The bacterial population of *E. coli* significantly reduced in 2 (4.11 vs. 6.59 $\log_{10}$ cfu/ml) ($p = 0.03$), 10 (7.28 vs.10.25 $\log_{10}$ cfu/ml) ($p = 0.01$), and 24 (7.11 vs. 11.48 $\log_{10}$ cfu/ml) ($p = 0.01$) h with sage oil and in 10 (6.3 vs. 10.25 $\log_{10}$ cfu/ml) ($p = 0.01$) and 24 (7.44 vs. 11.48 $\log_{10}$ cfu/ml) ($p = 0.01$) h with savory oil. The population of *S. aureus* significantly decreased with sage and savory oil in 4 (5.94 and 5.27 vs. 8.47 $\log_{10}$ cfu/ml, respectively) ($p = 0.02$), 10 (6.32 and 6.38 vs. 9.4 $\log_{10}$ cfu/ml, respectively) ($p = 0.02$), and 24 (6.46 and 7.3 vs. 10.53 $\log_{10}$ cfu/ml, respectively) ($p = 0.01$) h. Also, the sage and savory oil significantly decreased bacterial population of *S. agalactiae* in 4 (6.2 and 6.81 vs. 9.44 $\log_{10}$ cfu/ml, respectively) ($p = 0.01$), 10 (7.22 and 6.52 vs. 10.59 $\log_{10}$ cfu/ml, respectively) ($p = 0.01$) and 24 (6.74 and 6.5 vs. 11.79 $\log_{10}$ cfu/ml, respectively) ($p = 0.03$) h.

Table 1.
Chemical composition (relative % of peak area) of essential oil of sage determined by GC-MS analyses.

No.	Components	Retention time (min)	Area sum%
1	1R-α-Pinene	4.117	7.88
2	o-Cymol	5.909	3.61
3	Eucalyptol	6.092	4.57
4	γ-Terpinene	6.676	2.52
5	Thymol	12.664	20.41
6	Carvacrol	12.935	61.01

Discussion

Mastitis is a serious and prevalent problem and does not have effective treatments in organic dairy cattle. There is a need for organic antimicrobials for the production of organic foods and the prevention of antibiotic resistance [8]. Herein, we investigated the antibacterial property of savory and sage essential oils on three bacteria responsible for this pathology.

Considerable studies have been done on the antimicrobial effects of essential oils using model broth systems. The antimicrobial activity of plant extracts decreased when used in complicated systems or foods [2]. The antimicrobial activity of plant-derived molecules in laboratory media is significantly greater than that in complicated foods such as dairy products, fish, meat, and vegetables [9]. Similarly, the composition of milk, especially the amount of fat, reduced the antimicrobial effect of eugenol in milk [10]. According to these reports, the present study evaluated the MBC and MIC of the oils on the mastitis bacteria in milk instead of laboratory medium.

Thirteen constituents were recognized in the *S. hortensis* oil in this research. The most abundant chemical constituents in *S. hortensis* oil were eucalyptol (1,8-cineole) (32.45%), carvacrol (28.24%), α-pinene (13.42%), and thymol (9.71%). Major constituents of savory were reported carvacrol (41%), p-cymen (10%), and thymol (10%) from Bosnia Herzegovina [11]. Jafari et al. [7] analyzed essential oils of savory from Ardebil province of Iran, and 25 components were shown, and major oil components were γ-terpinene (37%), carvacrol(32%), and p-cymen (13%) while in a sample from Shiraz province of Iran, 22 compounds were identified and major com-

Table 2.
Chemical composition (relative % of peak area) of essential oil of savory determined by GC-MS analyses.

No.	Components	Retention time (min)	Area sum%
1	α-Pinene	4.117	13.42
2	m-Cymene	5.909	3.54
3	p-Menth-8-en-1-ol, acetate	6.018	2.3
4	Eucalyptol	6.099	32.45
5	γ-Terpinene	6.676	1.01
6	Linalool	7.66	2.58
7	(+)-2-Bornanone	8.889	1.41
8	Isoborneol	9.324	1.06
9	p-Menth-1-en-4-ol, (R)-(-)-	9.765	1.42
10	α-Terpineol	10.138	1.49
11	Linalyl acetate	11.554	1.38
12	Thymol	12.636	9.71
13	Carvacrol	12.874	28.24

Table 3.
MIC (%V/V) and MBC (%V/V) of savory and sage essential oils against tested bacteria

Bacterium	Savory		Sage		Savory+ Sage (1:1)	
	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	2.5	5	1.25	2.5	1.25	2.5
S. aureus	2.5	5	0.625	1.25	1.25	2.5
S. agalactiae	1.25	2.5	1.25	2.5	1.25	2.5

MIC: Minimum inhibitory concentration
MBC: Minimum bactericidal concentration

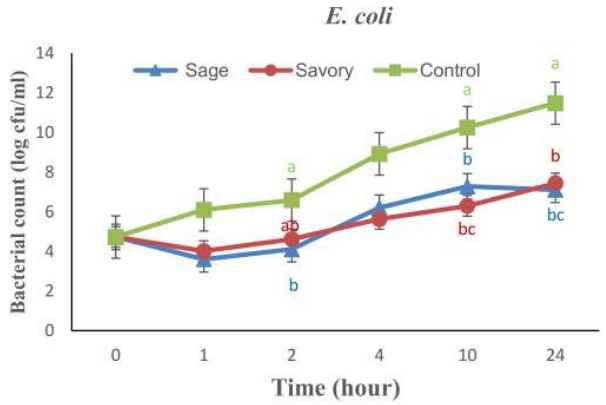

Figure 1.
Survival curve of *E. coli* in milk containing 0% (control, ■) and sub-MIC concentration of essential oil of savory (●) and sage (▲). [a-c] Values that are significantly ($p < 0.05$) different within the same time are indicated by different letters.

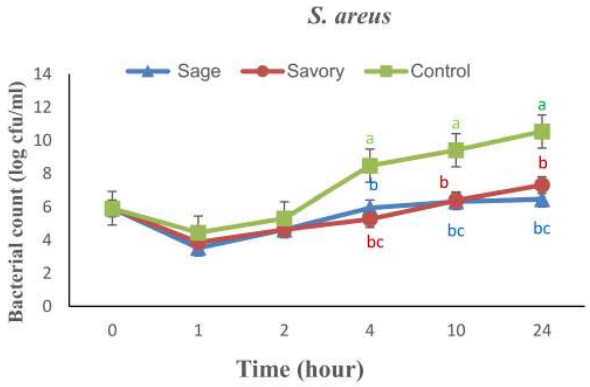

Figure 2.
Survival curve of S. aureus in milk containing 0% (control, ■) and sub-MIC concentration of essential oil of savory (●) and sage (▲). [a-c] Values that are significantly ($p < 0.05$) different within the same time are indicated by different letters.

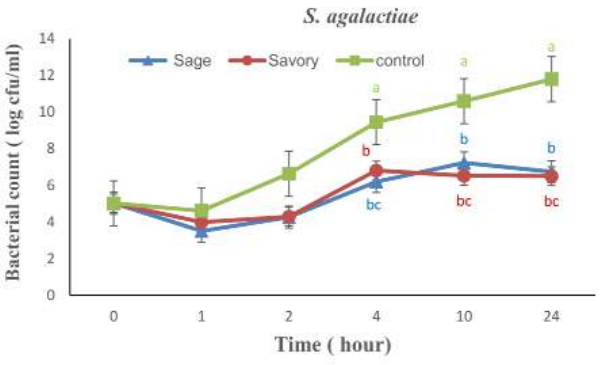

Figure 3.
Survival curve of S. agalactiae in milk containing 0% (control, ■) and sub-MIC concentration of essential oil of savory (●) and sage (▲). [a-c] Values tht are significantly ($p < 0.05$) different within the same time are indicated by different letters.

ponents were carvacrol (54%) and γ-terpinene (26%) [6]. In another study in Iran, major constituents of savory were reported carvacrol (56%), γ-terpinen (24%), and p-cymen (5%) [12]. In our study, eucalyptol (32.45%) was higher and γ-terpinen (1.01%) was lower than other studies while other components somewhat similar to other works.

In the present study, six compounds were recognized in the *S. officinalis* oil, and the main constituents were carvacrol (61.01%), thymol (20.41%), and 1R-α-pinene (7.88%). In one study, sage was raised at 18 experimental places in Italy, and major constituents were α-thujone, camphor, borneol, c-muurolene, and sclareol [13]. In another study from Italy, the primary substances of sage were cis-thujone (23.90%), camphor (19.22%), and 1.8-cineole (10.62%) [14]. Aumeeruddy-elalfi et al. [15] reported that the Mauritius sage EO mainly consists of thujone, followed by camphor and aromadendrene. Compositions of sage in the present work were somewhat different from most researches and amount of carvacrol + thymol was high that both compositions have potent antibacterial effect.

The variation in the content and composition of oils in different studies may be due to species, growth stages, plant origin and adaptive metabolism, conditions of climate, drying, distillation, and the part of the plant being analyzed [16].

Savory and sage oils had antibacterial activity against the bacteria in the present study, and we showed that sage and savory oil could kill all tested bacteria. The MIC and MBC of sage oil for *S. aureus* was lower than that of savory and savory+ sage. The MIC and MBC of three groups were similar for *S. agalactiae*. Sage oil exhibited stronger activity than savory and savory+ sage oil.

Most researchers declared that the MIC is a measure of the antimicrobial activity of Eos [9]. Different values have been reported for MIC and MBC of savory and sage. Jafari et al. [7] obtained MIC and MBC values of 0.01% and 0.8 % against *E. coli* and 0.2% and 0.2% against *S. aureus* for savory, respectively. MIC and MBC values of 0.03% and 0.1% against *E. coli* and 0.03% and 0.06% against *S. aureus* for savory have been reported, respectively [6]. In another study, the MIC value of savory for *E. coli* was obtained to be 5% [17].

Regarding sage, obtained MIC and MBC values of 5% against *S. aureus* and 0.8% against *E.coli* reported by Raffaella et al. [14] and Moghimi et al. [18], respectively. Also, a MIC value of 0.2% has been reported against *E. coli* and *S. aureus* for sage [15]. Moreover, a MIC value of 0.03% against *E. coli,* and MIC and MBC values of 12.5% and 6.1% have been reported for sage [5,19].

The different values of MIC and MBC in various studies might be because of the variable components of EOs and susceptibility of strain. Moreover, our MIC and MBC values are more than most of these researches which might be due to different media used in studies (synthetic medium of others versus milk of ours). The MBC/MIC ratio is used to determine the antimicrobial activity of EOs. The ratio of greater than 4 shows bacteriostatic and lower or equal to 4 shows the bactericidal characteristics [16]. This ratio showed a bactericidal effect of the oils on the strains tested.

In the present study, the miscibility and antimicrobial activity of savory and sage essential oils were tested in milk to obtain useful information for their application in bovine mastitis. Furthermore, a time-kill curve set of experiments was conducted to determine the time of inhibition or elimination of these pathogens. Savory and sage oils at sub-MIC concentrations caused a ~4.0 $\log_{10}$ cfu/ml reduction of *E. coli*, *S. aureus*, and a ~5.0 $\log_{10}$ cfu/ml of *S. agalactiae* reduction within 24 h. Significant reduction of bacteria started 2 (*E. coli*) or 4 (*S. aureus* and *S. agalactiae*) h after exposure to sub-MIC concentration. Higher concentrations may result in an early reduction of the bacterial population.

Essential oils have different components, and their antimicrobial activity cannot be contributed to one compound [20]. The antibacterial effect of essential oil may be attributed to the main compounds of essential oil and the synergism between main and minor compounds [21].

In this study, compounds such as eucalyptol (1,8-cineole) (a monoterpene hydrocarbon), carvacrol, thymol, and α-pinene (oxygenated monoterpenes) contributed to more than 70% of the chemical composition of oils. Oxygenated compounds, especially phenolic compounds as carvacrol and thymol, have higher antibacterial activity, while hydrocarbon monoterpenes possess the lowest potential because they have limited diffusion through the medium due to their low water solubility [22]. In this study, sage oil exhibited stronger activity than savory, an effect which might be due to a higher amount of stronger antibacterial components (carvacrol+ thymol) in sage (81.42%) than savory (28.24%).

In conclusion, the essential oil of sage and savory had antibacterial activity, and sage had higher activity than savory on the mastitis-causing bacteria (*S. aureus, S. agalactiae*, and *E. coli*). Sage EO might be effective in the treatment of mastitis as an alternative or adjunct to antibiotics. However, further *in vivo* tests are needed to evaluate the efficiency on the treatment of bovine mastitis and potential side effects on the mammary gland tissue.

Materials & Methods

This study was performed in Gonbad Kavous University (Gonbad Kavous, Iran) from October 2017 to June 2018.

Chemical composition analysis of the essential oils

Essential oils of sage and savory were purchased from Dorrin Golab Co. (Kashan, Iran). Gas chromatography/mass spectrometry (GC/MS) analysis was performed employing a gas chromatograph connected with a mass detector (Model 5977A, Agilent Technologies, USA) and equipped with an HP-5MS capillary column (phenylmethyl siloxane, 30 m × 0.25 mm ID 0.25 um, Agilent Technologies). The temperature of the injector was 270 °C, and the temperature of the oven was raised from 60 °C (0 min) to 200 °C by a rate of 5 °C/min. The analysis was performed using helium as a carrier gas, while the flow rate was adjusted to 1 mL/min and injection volume (1 ul). The interface temperature was set at 280 °C, and the mass range was 35 - 500 m/z.

Bacterial strains

The activity of the EOs was tested toward three different major mastitis pathogens, including *Staphylococcus aureus* (PTCC 1113), *Streptococcus agalactiae* (PTCC 1768), and *Escherichia coli* (PTCC 1399). These bacteria were obtained as a lyophilized culture from Persian Type Culture Collection, Tehran, Iran (PTCC). The lyophilized cultures were grown twice in tubes contaning 10 ml of tryptic soy broth (TSB) (Biolife, Milano, Italy) at 37 °C for 18-20 h (overnight). Sterile glycerin (1:5) was used to dilute the cultures, and then the cultures were stored in microtubes at -20 °C. To achieve fresh bacterium, it was cultured twice in TSB at 37 °C for 20 h followed by streaking on tryptic soy agar (TSA) (Biolife, Milano, Italy) slants and incubation under the same conditions. The cultures were stored at 4 °C and sub-cultured monthly [23].

Preparation of Inoculum

Cells from working cultures were transferred to tubes of TSB to obtain bacterial inoculum. The cultures incubated 18 h at 35 °C, and then second subcultures were provided. The bacterial broth cultures were adjusted to optical density (OD) (absorbance) of 0.1 at 600 nm, using a spectrophotometer (Libra S12, Biochrom Ltd., Cambridge, London). These adjustments gave a cell concentration of 2.4×10^{11} cfu/ml for *E. coli*, 3.4×10^{10} cfu/ml for *S. aureus*, and 1.64×10^{11} cfu/ml for *S. agalactiae*. The cell counts in the suspensions were estimated by duplicate plating from tenfold serial dilutions on TSA and counting the colonies after 24 h incubation at 35 °C [24].

Milk Preparation

Raw milk with no antibiotic residues was collected and autoclaved at 121 °C for 15 min.

Determination of MIC and MBC

1/2 dilution of the oils was made with dimethyl sulfoxide (DMSO, Sigma, Germany) to increase solubility in the culture medium and filter sterilized. This dilution was used in the antibacterial analysis. Herbal oils alone or in combination (1:1) were investigated according to a modified protocol for broth dilution testing [25]. Autoclaved milk was utilized rather than synthetic broth medium as the growth medium. Twofold serial dilutions (10, 5, 2.5, 1.25, and 0.625 %) of the oil were performed for the determination of MIC. Treatments were added to milk, then vials were vortexed for 90 s. The total volume of test vials was 1 mL

of liquid. Afterward, 100 µl of 1:300 dilution of inoculum of each bacterium was inoculated into each tube. The vials were vortexed for 15 s and incubated for 24 h at 37 °C. Following incubation, vials were vortexed for 15 s. Bacterial counts were determined with serial dilution employing a 0.1-mL aliquot of the vortexed vial and sterile normal saline to create eight 10-fold dilutions. Dilutions were plated on eighths of a TSA plate and incubated for 24 h at 37 °C. Bacterial populations of dilutions were counted. The occurrence of synergism/antagonism in antibacterial action between the essential oils of sage and savory was tested with mixing of oils volume to volume.

Several controls were in treatments. Milk was a negative control to evaluate autoclavation. Milk + bacteria was a positive control to check bacterial growth in the milk. A positive control containing the bacterial culture and DMSO without the EO was performed as well. MBC was the lowest concentration that inhibited bacterial growth following subculture on TSA. Ante-MBC concentration was taken as the MIC. Each experiment was repeated twice on at least two separate occasions and was repeated if results differed by more than one doubling dilution.

Growth curve of bacteria

Sterile milk containing the sub-MIC of EOs with each pathogen was inoculated in the same way as the above MIC tests to assess the bactericidal kinetics of oils. Control samples contained inoculated milk without EO. The samples were incubated at 37 °C for 24 h. Bacterial counts were enumerated in 1, 2, 4, 10, and 24 h of incubation by plating 0.1 mL portions of the samples with or without serial dilutions (1:10 in normal saline). Each experiment was done in duplicate. Bacterial counts ($\log_{10}$ cfu/ml) against time (hour) were plotted for time-kill curves construction.

Statistical analysis

All the tests were carried out in duplicate. The data were evaluated to a one-way analysis of variance (ANOVA) and *Tukey's* test using the SPSS statistical software (SPSS, Chicago, USA). p values < 0.05 were considered significant.

Authors' Contributions

R.R. and F.G. conceived and designed the experiment. S.Z. performed the experiments. R.R. and A.K. analyzed and interpreted the data. R.R. and F.G. drafted and critically revised the manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Trypanosoma brucei brucei is more pathogenic in rats compared to mice, making rats a better candidate for the relevant research studies

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ABSTRACT

Trypanosomosis is an economically important disease that has raised great and diverse kinds of research using different types of animals. Hence, this study is aimed at determining the better laboratory animal between the Swiss albino mice and Wistar albino rats in *Trypanosoma brucei brucei* studies. This study assessed the pathogenesis of *T. b. brucei* in Swiss albino mice and Wistar albino rats by probing the level of parasitemia, mean temperature, mean weight, hematological and histopathological parameters caused by the hemoprotozoan. Twenty laboratory animals, of mice (10) and rats (10) were grouped in two (control (5) and infected (5)), with the infected group inoculated with the blood protozoan intra-peritoneally. *Trypanosoma b. brucei* was detected in the blood of both laboratory animals on day one post-infection, with all the infected animals dying between day seven and eight post-infection. The protozoan exerted a significant ($p < 0.05$) effect on the mean temperature, mean weight, and hematological parameters of the infected animals. Pathological effects of *T. b. brucei* infection were seen in the liver and lungs of mice, and the liver, lungs, kidney and spleen of rats. The pathogenesis of *T. b. brucei* was more severe in rats compared to mice based on the studied parameters. These findings showed that rats are better candidates for *T. b. brucei* studies.

Keywords

parasitemia; pathogenesis; Swiss albino mice; Trypanosoma brucei brucei; Wistar albino rats

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Abbreviations

Hgb: Hemoglobin
PCV: Packed cell volume
RBC: Red blood cell
SEM: Standard error of the mean
T.: Trypanosoma
T. b.: Trypanosoma brucei
T. b. brucei: Trypanosoma brucei brucei
TWBC: Total White blood cell count

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Introduction

Trypanosomosis is one of the world's most important diseases of humans and animals that is caused by flagellated blood protozoans that belong to the family *Trypanosomatidae* [1,2]. Trypanosomosis is generally characterized by high levels of parasitemia, severe anemia, cellular infiltrations, marked changes in the lymphoid system, progressive emaciation, and often death [1,3]. A number of *Trypanosoma* species are known to cause the general trypanosomosis in animals and human, these include but not limited to *T. brucei, T. congolense, T. vivax, T. avium, T. suis, T. melophagium, T. melophagium, T. cervi, T. musculi* and *T. cruzi* [1,4].

Trypanosoma brucei is a model for trypanosome studies and one of the most important Trypanosoma species [4]. *Trypanosoma brucei* (*T. b.*) has several subspecies including *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. b. evansi* and *T. b. equiperdum*, with *T. b. gambiense* and *T. b. rhodesiense* been the causative agent for human African trypanosomiasis (HAT) [1,4,5]. *Trypanosoma b. brucei* is one of the *Trypanosoma* species responsible for causing nagana also known as African animal trypanosomosis (AAT) in a wide variety of animals [6]. It is a vector-borne *Trypanosoma* species that is being transmitted by tsetse flies (*Glossina* species) and it inhabits the blood plasma, intercellular tissues, and body cavity fluid of an infected animal, thereby causing anemia and tissue damage [3,7]. African animal trypanosomosis results in either an acute, subacute or chronic disease and it constitutes a serious threat characterized by intermittent fever, anemia, occasional diarrhea leading to a severe reduction in productivity, loss of weight, decreased milk yield, reduction in carcass quality and capacity for work, and even death due to the failure of animals to utilize available food efficiently [8,9]. Trypanosoma brucei is known to cause pathological lesions in the liver, spleen, kidney, lung, and hearts of domestic and wild animals [1].

Various studies on *T. b. brucei* have been conducted using laboratory mice [10,11] and albino rats [2,9] with satisfactory levels of results. This body of evidence is aimed at determining the more suitable laboratory animal between laboratory mice and albino rats for *T. b. brucei* studies by assessing the level of parasitemia, mean temperature, mean weight, and hematological and histopathological parameters between the two animal subjects when infected with *T. b. brucei.*

Results

After infection, *Trypanosoma* mean parasitemia and standard error of the mean (SEM) was determined. Parasitemia was seen on the first day post-infection in both mice and rats with the parasitemia of mice higher than that of rats but the difference was not significant ($p > 0.05$). The level of parasitemia increased progressively in both laboratory animals with parasitemia peaking on day 7 in mice and day 8 in rats. The difference in the total parasitemia level was not significant ($p > 0.05$) between the laboratory animals (Fig. 1).

There was no significant difference ($p > 0.05$) in the mean body temperature (°C) between the control and the infected mice until day 4 post-infection, while that of rats showed significant difference ($p < 0.05$) by day 3 post-infection (Figs. 2A and 2B). The mean weight of infected mice decreased progressively post-infection with the difference being significant ($p < 0.05$) by day 6 post-infection, while the reduction in the mean weight of rats was significant ($p < 0.05$) by day 3 post-infection (Figs. 2 C and D).

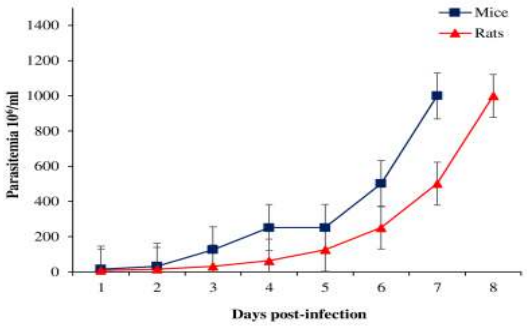

Figure 1.
T*rypanosoma b. brucei* parasitemia in mice and rats. Statistical analysis showed no significant differences ($p > 0.05$) for parasitemic effect between the two labouratory animals. Values are means ± SEM.

Trypanosoma b. brucei-infected mice started dying on the first day post-infection and they all died by day 7. Forty percent of them lived until day 4 post-infection. Rats infected with the protozoan started dying on day 1 post-infection and 60% of them survived until day 5 after which they all died by the 8th day (Table 1).

The mean PCV and hemoglobin concentration of infected mice and rats progressively decreased post-infection. The differences in the PCV and hemoglobin concentration between the control and the infected mice showed statistical significance ($p < 0.05$) at day 5 post-infection, while that of rats showed statistical significance ($p < 0.05$) at day 4 post-infection (Figs. 3A, 3B, 3C and 3D). There was no significant difference ($p > 0.05$) in the mean red blood cell (RBC) count between the control and the infected mice from day 1-4 post-infection until day 5 post-infection, when the RBC count was significantly lower ($p < 0.05$) in the infected mice compared to the mice in the con-

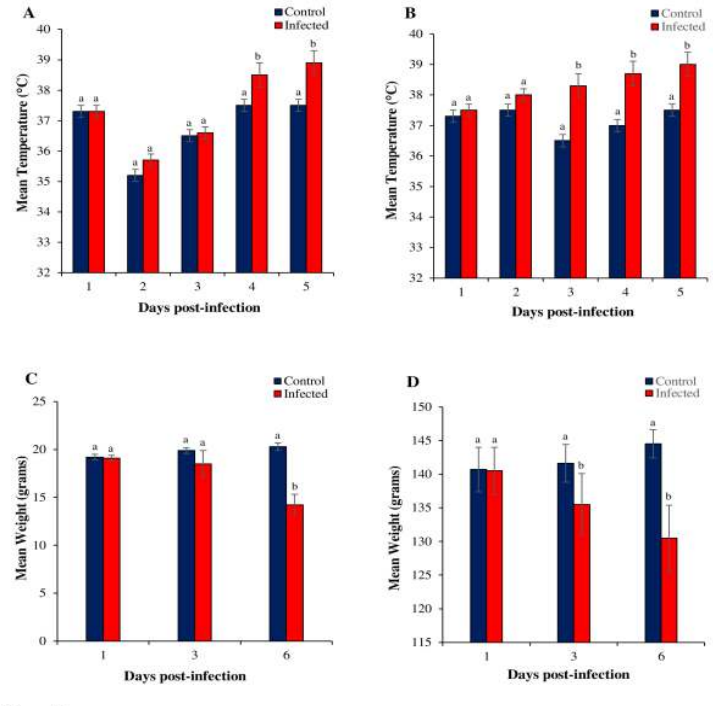

Figure 2.
Mean body temperature (°C) of mice (A) and rats (B); mean weight (grams) of mice (C) and rats (D) infected with *Trypanosoma b. brucei*. Each column represents the mean ± SEM. Different alphabets (a,b) in each day post-infection represents statistical significance at $p < 0.05$.

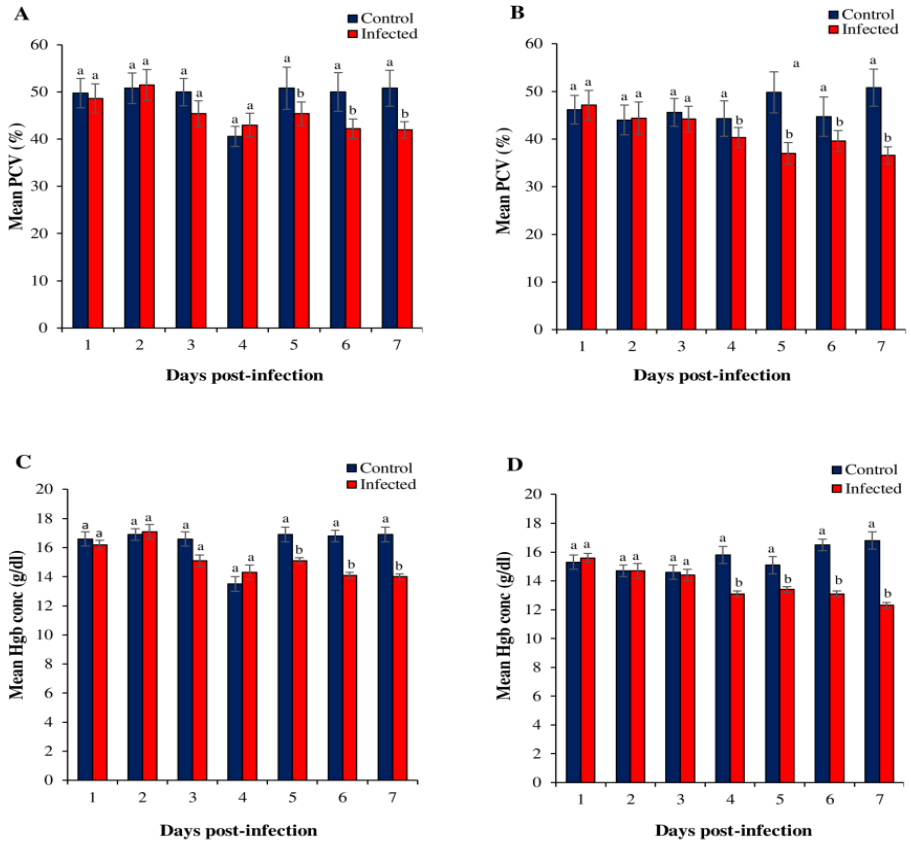

Figure 3.
Mean packed cell volume (%) of mice (A) and rats (B); mean hemoglobin concentration (g/dl) of mice (C) and rats (D) infected with *Trypanosoma b. brucei*. Each column represents the mean ± SEM. Different alphabets (a,b) in each day post-infection represents statistical significance at $p < 0.05$.

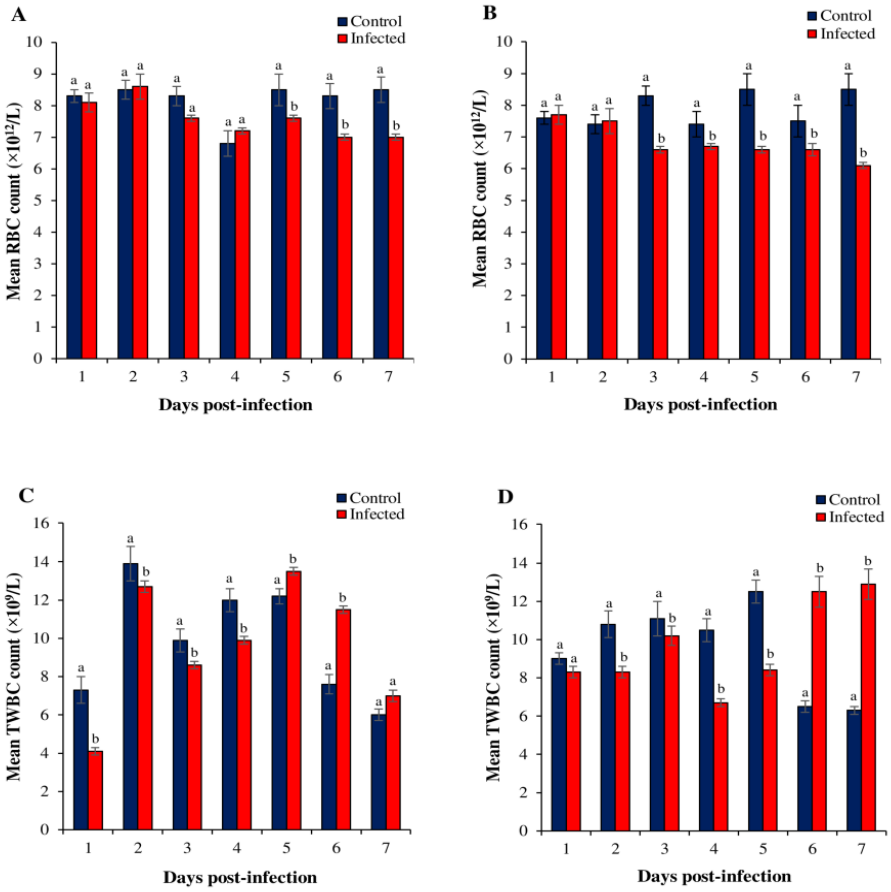

Figure 4.
Mean red blood cell count (×10^{12}/L) of mice (A) and rats (B); mean total white blood cell count (×10^{9}/L) of mice (C) and rats (D) infected with *Trypanosoma b. brucei*. Each column represents the mean ± SEM. Different alphabets (a,b) in each day post-infection represents statistical significance at $p < 0.05$.

Table 1.
Kaplan-Meier mean survival time of *Trypanosoma b. brucei* infection in mice and rats.

Groups	Mean survival time (days)
Mice infected with *T. brucei*	4.7[a]
Mice (control)	8.0
Rats infected with *T. brucei*	5.4[a]
Rats (control)	8.0

[a] = significant at $p < 0.01$ within each animal species.

trol group (Fig. 4A). A similar finding was observed in rats with a significant difference ($p < 0.05$) seen from day 3 post-infection (Fig. 4 B). The mean total white blood (TWBC) count of the infected mice was initially significantly lower ($p < 0.05$) than that of the control group until day 4 post-infection, between days 5 and 6, the TWBC count becomes significantly higher in infected compared to the control ($p < 0.05$), while by day 7 it was not significantly different ($p > 0.05$) (Fig. 4 C). Fig. 4 D shows the mean TWBC count between the control and infected rats. The mean TWBC count was consistently lower in the infected group compared to the control group until day 6 post-infection when it reversed. The difference was significant ($p < 0.05$) from day 2-7 post-infection.

The histopathological sections of the livers of the infected groups of mice revealed livers with congested central veins and perivascular cuffing (Fig. 5A), while that of rats showed hypertrophy of the hepatic cords with obstruction of the sinusoids (Fig. 6A). The histopathological sections of the lung of the infected mice showed congestion in the blood vessels, with airways edema (Figs. 5B), and expansion of the interstitial, with emphysema in infected rats (6B). Significant histopathological changes were observed in the kidney sections of *T. b. brucei*-infected rats compared to the uninfected rats, and this was characterized by congested intertubular space (Fig. 6C). Histopathological sections of the spleen of infected rats showed depletion of the lymphoid follicles (Fig. 6D). There were no histopathological lesions seen in the kidney and spleen of infected mice.

Discussion

The establishment of parasitemia in all the infected mice and rats is in line with previous studies done

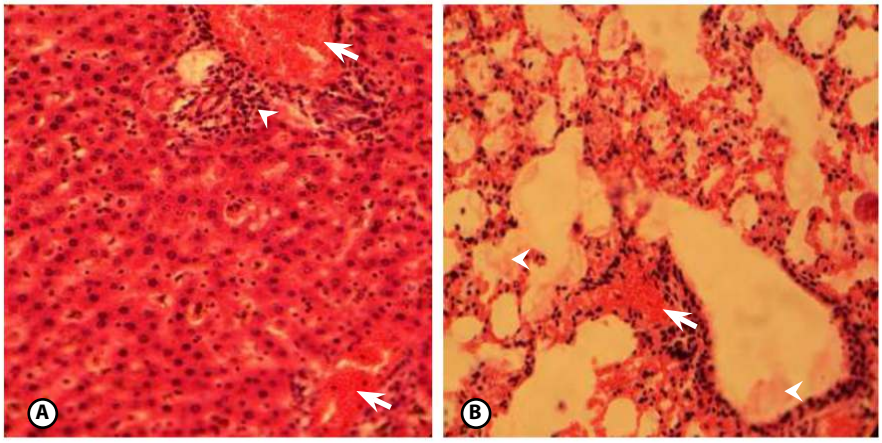

Figure 5.
A) Liver, congestion of the central veins (arrow), perivascular cuffing (arrowhead). B, Lung, congestion in the blood vessels (arrow), with airways edema (arrowheads) of mice infected with *Trypanosoma b. brucei* (H&E, original magnification 400X).

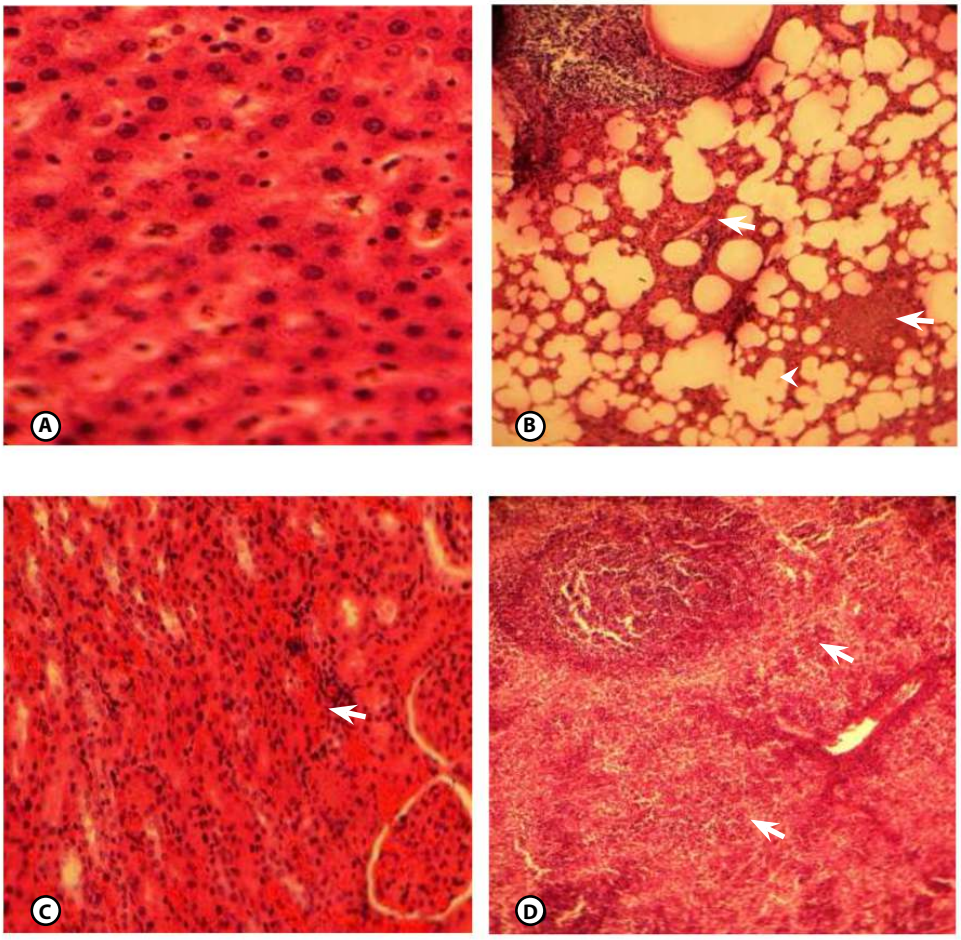

Figure 6.
A) Liver, hypertrophy of the hepatic cords with obstruction of the sinusoids. B) Lung, expansion of the interstitial tissue (arrows), with emphysema (arrow head). C) Kidney, congested intertubular space (arrow). D) Spleen, depletion of lymphoid follicles (arrows) of rats infected with *Trypanosoma b. brucei* (H&E, original magnification 400X).

on the infection of *T. b. brucei* in laboratory animals [2,11,12]. The detection of the protozoan in the peripheral blood immediately after infection (first day post-infection) was investigated in studies by Udensi and Fagbenro-Beyioku [10] and Ademola and Odeniran [13] who detected *T. b. brucei* in the blood of mice 2 days and 9 days post-infection, respectively. In studies conducted by Egbe-Nwiyi et al. [2] and Habila et al. [3] the presence of the hemoprotozoan in the blood of albino rats was confirmed 7 days and 4 days post-infection, respectively. Factors such as the strains and virulence of the isolates (*T. b. brucei*), the immune status, nutritional requirement, and degree of susceptibility of the hosts to the hemoprotozoan may have resulted in the varied onset of the parasitemia. The progressive nature of the parasitemia level in both mice and rats attests to the invasive nature of the protozoan.

We observed that all the mice died by the 7th day post-infection, with rats all dying by the 8th day. Similarly, Udensi and Fagbenro-Beyioku [10] reported a 100% mortality with a mean survival time of 5 ± 1 in

rats infected with *T. b. brucei*. Death due to *T. b. brucei* infection is associated with congestive heart failure which occurs as a result of anemia and myocarditis in infected hosts [1].

Trypanosoma *b. brucei* is associated with pyrexia in infected animals [1,14], this supports the increase in mean body temperature that was observed in our study.

Loss of appetite (anorexia) is a vital clinical sign associated with *T. b. brucei* infection in animals, and this ultimately leads to weight loss and emaciation [15] which was observed among the infected mice and rats in our study. Ademola and Odeniran [1] reported a significant weight loss among mice infected with *T. b. brucei* compared to uninfected mice, while Habila et al. [3] observed a similar finding among rats infected with the hemoparasite compared to those not infected.

The significant decrease in PCV, Hgb concentration, and RBC count we observed in both infected Swiss albino mice and Wistar albino rats suggest the presence of blood loss resulting to anemia, which is associated with an increase in the level of parasitemia of *T. b. brucei* infection. The release of hemolytic factors into the infected host's blood by the dead trypanosomes destroys the red blood cells, thereby leading to a reduction in PCV, Hgb, and RBC count [16,17]. Notably, a significant decrease in these parameters was first observed in rats compared to mice. Our observation is consistent with previous reports by Nwoha and Omamegbe [7], Ukpai and Nwabuko [9], and Ademola and Odeniran [13].

The increase in TWBC count in the latter days of the infection in both the infected mice and rats indicates the presence of advanced infection. In response to this infection, the body employs its immune arsenals to fight the invading *T. b. brucei* and this process of immune response will boost the production of a high number of WBC [9, 18], which is similar to our findings.

The histopathological lesions seen in the tissues and organs are a result of *Trypanosoma b. brucei* infection. *Trypanosoma b. brucei* infection in animals is known to cause immunoproliferative disorder of B-lymphocytes and plasma cells. Disorder in these cells may either directly or indirectly be responsible for the impaired functions of various organs [1,19].

The effect of *Trypanosoma b. brucei* infection was more severe in rats compared to mice, and the histopathological lesions were seen more in the organs of rats than the organs of mice. This showed that rats have more overlapping clinical and pathological data that are seen in *T. b. brucei* infection in animals, compared to that of mice. The suitability of an animal model for infectious disease studies is dependent on how well the animal model shows a sufficient amount of overlapping data that are seen in the humans biological (physiological, pathological, and pharmacological) information matrix [20]. In a similar study, Muchiri et al. [21] reported that *Trypanosoma brucei rhodesiense* caused more prominent clinical and pathological lesions in *Mastomys natalensis* rats compared to Swiss white mice, and concluded that *Mastomys natalensis* was a suitable model for studying the pathophysiology of human African trypanosomiasis.

Trypanosoma b. brucei induced noticeable pathogenesis in both Swiss albino mice and Wistar albino rats. There was an earlier significant increase in the mean body temperature and weight loss, and an earlier significant decrease in PCV, Hgb concentration, and RBC count in the infected rats compared to infected mice. There was an earlier significant increase in the TWBC count in infected mice compared to infected rats. The pathogenesis of *T. b. brucei* was manifested more in the organs of rats (liver, lungs, kidney, and spleen) compared to the organs of mice (liver and lungs). These findings, therefore, showed that *T. b. brucei* is more pathogenic in rats compared to mice, making rats a better candidate for *T. b. brucei* studies.

Materials & Methods

Experimental animals and grouping

Ten (10) male and female Swiss albino mice, 7–8 weeks of age and weighing between 15 and 18 grams, and 10 Wistar albino rats (of both sexes, 9–11 weeks of age and weighing between 127 and 131 grams) were acquired from the faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The mice and rats were housed in plastic cages covered with wire mesh and allowed to acclimatize for 2 weeks before the onset of the experiment. The animals were maintained on commercial grower's poultry feed, maize bran, and groundnut cake, compounded in a ratio appropriate for the type of laboratory animals. Water was provided ad libitum. The animals (mice and rats) were divided into two groups of five animals each (Group A = the control; and group B = the infected).

Source and infection of T. b. brucei

The *T. b. brucei* used for the study was obtained from the Nigerian Institute for Trypanosomiasis Research (NITR), Kaduna, Nigeria. It was originally isolated from a natural infection in cattle, in Kaduna State. It was confirmed to be *T. b. brucei* using feautures described by Taylor et al. [1]. The *T. b. brucei* was inoculated into Wistar albino rats and transported to the Protozoology Research Laboratory, Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, for subsequent studies.

For the purpose of infections, infected blood with *T. b. brucei* was obtained from infected donor rats. The parasite populations were counted and diluted in phosphate buffer saline. The laboratory animals were inoculated intraperitoneally with 0.20 ml of *T. b. brucei*-infected blood estimated to be 1×10^5 parasites/ ml using the hemocytometer.

Parasitemia, body temperature, body weight and survival assessment

The infected Swiss albino mice and Wistar albino rats were screened for trypanosomes at 24 h interval post-infection using the wet blood mount technique as described by Taylor et al. [1]. A drop of blood was placed on a clean glass slide, and a cover glass slip was placed over it. The quantity of blood was just insufficient to fill the whole space under the cover glass slip when this was pressed down gently. The film was examined under 400X magnification of a light microscope (Olympus®, Japan). A field was chosen in which the cells are evenly distributed. The rapid matching counting method as described by Herbert and Lumsden [22], was used to assess the number of *T. b. brucei*. Briefly, the microscopic appearance of a wet film, that has more than one *T. b. brucei* parasite was matched with one of a series of eight pictures of microscopic fields in the chart and table for estimating trypanosome parasitemias of Herbert and Lumsden [22]. The value in the box of the corresponding charts and the tables was used as the logarithm of the number of *T. b. brucei* per ml. The rectal temperature for each experimental animal was assessed daily at 7.00am for 5 days, using the digital thermometer. A portable weighing scale (Atom Digital Precision A-110C, China) was used to determine the weights of the animals at 72 h interval during the course of infection. Survival was determined by daily inspection post-infection with the trypanosome and Kaplan-Meier survival curve was used in calculating the mean survival time.

Hematological analysis

Blood sample for hematological studies was collected from the median cantus of the eye of each infected and non-infected mice and rats. Hematological analysis was carried out using standard protocols [23]. The total white blood cell (TWBC) count was determined by using a coulter counter (Cyan Hemocytometer, Belgium) as described by Cheesbrough [24].

Histopathological examination

Tissue samples collected from the liver, lungs, kidney and spleen were preserved in 10% buffered neutral formalin (BNF). After 48 hours of fixation, the tissue samples were processed (washed in 50% and 70% alcohol), embedded in paraffin wax and sectioned at 5 microns using a microtome. The sections were mounted on clean grease-free glass slides and stained with Hematoxylin and Eosin (H&E) stains as described by Luna [25]. The stained slides were examined microscopically using the 40X objective (400X magnification). The tissue samples were assessed using standard protocols. Histopathological lesions were observed, recorded and photo-micrographed with the aid of a digital camera (AmScope MT300 3.1MP, made in The United States of America).

Statistical analysis

Statistical analysis was conducted using the One-way Analysis of variance (ANOVA). *Tukey's* multiple comparison test was used as Post-Hoc Test. The test was used to measure the differences in the various parameters within the different groups. Statistical significance was set at 5% ($p < 0.05$). GraphPad Prism Version 5.0 for Windows (GraphPad, San Diego, CA, USA) was used for the statistical analysis. The mean survival time was calculated using the Kaplan-Meier survival curve.

Ethics approval

The study protocol was approved by the Research and Ethical Committee of the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. International, national, and/or institutional guidelines for the humane use and handling of laboratory animals were adequately followed.

Authors' Contributions

KH, IAL, and SA conceived and planned the experiments. KH, IAL, and SA carried out the experiments. KH and IAL contributed to sample preparation. KH, SDO and SAA contributed to the interpretation of the results. SDO took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Histomorphometric analysis of mature female Japanese quail (*Coturnix coturnix japonica*) stomach

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ABSTRACT

The study analyzed the histomorphometry of the mature female Japanese quail (*Coturnix coturnix japonica*) stomach with the aid of ImageJ software. The different histological parts were identified using a compound microscope. Five mature laying female Japanese quail were collected and necropsied. The digestive organs, particularly proventriculus and gizzard, were collected and processed for tissue staining. Histological identification and measurement of thickness and depth of various structures were subsequently performed. Comparable to other avian species, the proventriculus was comprised of four layers: thin tunica serosa (22.69 µm), tunica muscularis (235.07 µm) with outer longitudinal and inner circular smooth muscle layers, thick tunica submucosa (2,164.37 µm) containing glands, and innermost tunica mucosa (553.42 µm) with papillae. The gizzard was characterized by four tunics: thin tunica serosa (60.44 µm), thick tunica muscularis (1,480.07 µm), tunica submucosa (112.25 µm), and tunica mucosa (456.15 µm) where the glands, crypts, and koilin can be found. The findings suggest that the histology of proventriculus and gizzard of the Japanese quail have no remarkable difference compared to other poultry species. However, the histomorphometry of the organs examined had remarkable variations as compared to other avians.

Keywords

gizzard; histomorphometry; Japanese quail; proventriculus

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Abbreviations

µm: micrometer
H & E: hematoxylin and eosin

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The poultry industry is one of the largest animal sectors in agriculture [1] and has rapidly evolved over the past 100 years from backyard household production to highly sophisticated commercial production units [2]. Nowadays, quails have gained particular attention because of their unique characteristics and as an alternative to chicken and duck eggs and meat. Quails sexually mature rapidly, have shorter incubation periods, are fast-growing and efficient food converters and prolific egg producers, therefore making them the most suitable and effective poultry [3]. Even though they are the smallest birds used for commercial production, quail production is still economically viable and technically feasible, in addition to its resistance to various diseases [4]. They have attained economic importance in the poultry industry through the unique flavor of their eggs and meat [5].

An ideal functioning digestive system is required as commercial poultry breeds are expected to efficiently utilize feeds [6]. The most active part of the avian digestive system is the stomach [7]. In birds, the stomach is divided into two distinct parts [8], the glandular portion (proventriculus) and the muscular portion (ventriculus or gizzard) [7].

The proventriculus or pars glandularis is the cranial compartment of the avian stomach which is functionally similar to the mammalian stomach. It is a relatively small tubular organ and is elliptical [9–12]. Grossly, it is situated caudal to the crop and connected to the gizzard [12,13]. It is responsible for the secretion of pepsinogen, hydrochloric acid (HCL), and the zymogenic component of gastric juice that is needed for the digestion of feeds [7–11,14].

The gizzard or the ventriculus is the muscular compartment of the avian stomach and incomparable to the mammalian stomach [9]. It is small and round in shape with tapering ends situated caudal to the proventriculus and relatively located between the lobes of the kidney and partly behind the left lobe of the liver [7,15]. In granivores, insectivores, and herbivores, the ventriculus is well-developed and distinct from the proventriculus [9]. This mechanical stomach is responsible for providing a suitable environment for the physical and chemical reduction of the bird's intricate nutritional diet [9,10,16].

Currently, there are limited researches on Japanese quail that can help us understand its microscopic anatomy. Many researchers prefer to use other species of birds such as chicken, ducks, and turkey due to the ease of collecting and visualizing their organs. Histomorphometric studies on the organs of Japanese quail are not given enough emphasis as compared to their egg and meat production aspects and traits. Hence, in the present study, histologic parts of the quail stomach were identified and measured. In addition, the study used mature female Japanese quails (more than six weeks of age) raised in the backyard for egg production, and no attempt was performed in determining the genetic purity of the quail or associate the management practices being implemented in the farm in the evaluation of the organ.

Proventriculus

Figure 1 shows the different histological parts of the proventriculus, particularly the four layers of the organ. These layers were also documented in various species of birds [7,17-21]. The tunica mucosa is the innermost layer consisted of numerous microscopic invaginating folds that varied in size and height, and arranged concentrically around the single duct that opens in the lumen [10,11,20,22,23].

The surface epithelium was a simple columnar type with cells having vesicular nuclei located near the basement and relatively pale staining cytoplasm (Figure 1 – upper left and upper right) that produce mucous secretion [7,10,11,17,18,20,22,24]. The lamina propria was constructed by loose connective tissue and blood vessels (Figure 1 – upper left and upper right). It is also constructed by lymphatic infiltration and tissues; the numerous blood vessels around the glandular epithelium were capillaries [12,18–20,22,24]. The lamina also extended inside the mucosal folds and contained simple tubular glands that opened into the lumen of the organ (Figure 1 – upper left) that produce mucous secretions [7,11].

The inner surface of the proventriculus displayed raised low and wide papillae on the lumen that serve as exit ducts of the composite gland for protein digestion and secretion of digestive juices [18,19]. Functionally, the tunica mucosa serves as a railway to the lumen of the proventriculus to convey the essential enzymes and secretions that are important in the digestion of feeds as well as the protection and absorption of nutrients [9].

The tunica submucosa comprised the bulk of the proventriculus since it catered the proventricular lobes and glands which formed the utmost thickness of the proventricular wall (Figure 1 – upper left). Similar to previous studies [11,13,17,18,22,23], the compound branched tubuloalveolar proventricular glands were multilobular in structure and arranged in pyramidal or conical shape (Figure 1 – upper left). The glands are characterized by oval, rounded, hexagonal, elongated, conical, or polymorphic lobules and are divided by a thin perilobular areolar connective sheath composed of fibroblast and smooth muscle fibers rich in blood vessels. The glands contain numerous secretary alveoli or tubules that open together in a wide central cavity, and ducts from several lobules merge together to form a short main duct that connects to the mucosal

papillae and opens in the lumen of the proventriculus [10,11,17–20,23,24].

The secretory cells forming the simple cuboidal epithelium were oriented obliquely to the long axis of the tubules and were divided by a narrow space giving a serrated appearance (Figure 1 – upper left and upper middle). Such oxyntico-peptic cells possess regularly large and round nuclei which are located close to the basement of the cell. These cells are capable of secreting both hydrochloric acid and pepsinogen which aid in the better digestion and breakdown of feeds [10,18,20,23,24].

The tunica muscularis consisted of two layers with inner circular and outer longitudinal smooth muscle fibers (Figure 1 – upper left and upper middle). As observed in previous studies [7,10,11,17-20,24], the inner circular smooth muscle fiber was thicker and characterized by round basic cells scattered throughout the muscle bundles, while the thin outer longitudinal muscle was characterized by flat basic cells also scattered throughout. The tunica muscularis is accountable for the back-and-forth motility of feeds in the gizzard and proventriculus [9].

The tunica serosa is the outermost layer of the proventriculus consisting of smooth muscle fibers. including dense connective tissue in which nerves and blood vessels are distributed can also be found in this layer [7,11,18,19].

Table 1 shows the measurements of the microscopic parts of the quail proventriculus including its four main layers: the tunica mucosa, the tunica submucosa which is the thickest due to the numerous proventricular glands found in this layer, the tunica muscularis, and the tunica serosa which is the thinnest. It was noted that the tunica muscularis at the posterior section of the organ is relatively thicker than the anterior and middle sections. This may be attributed to the gradual transition of the organ as it connects to the muscularly structured gizzard.

In the study on striated scope owl [7], the mean thickness of the tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa was about five,

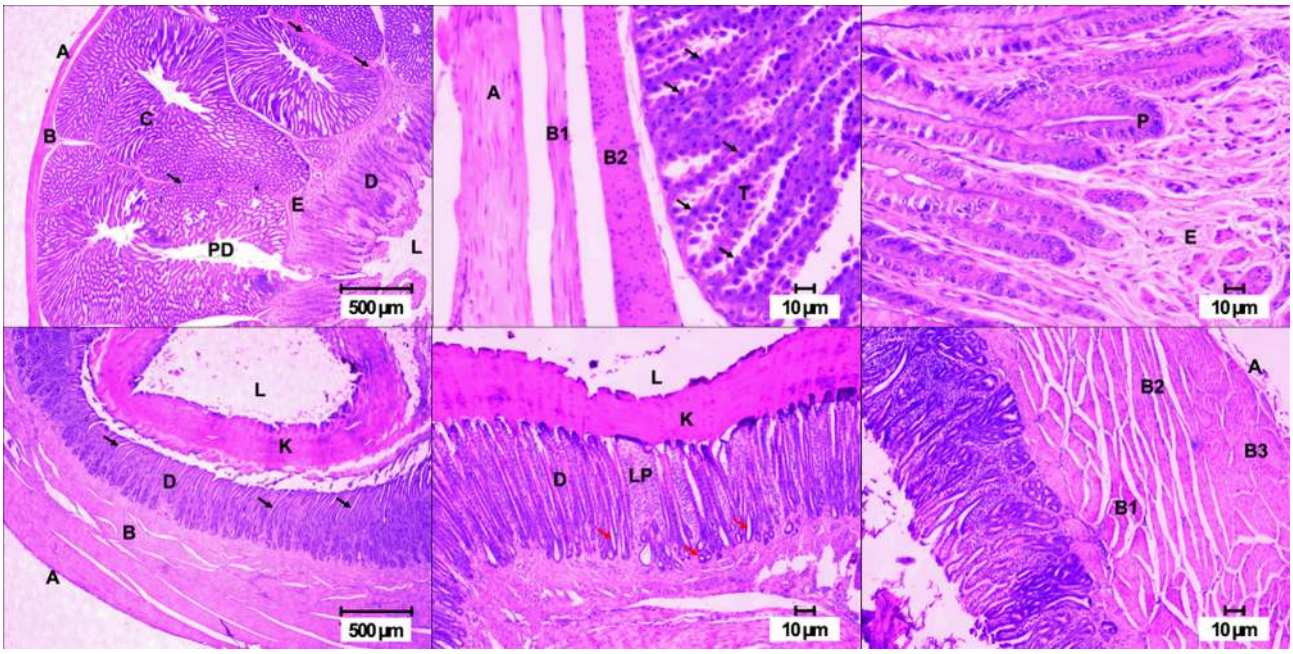

Fig 1.
Transverse sections of the proventriculus (upper) and gizzard (lower) mature female Japanese quail (*C. coturnix japonica*) stained with H & E at 40x magnification.
The proventriculus: (A) tunica serosa, (B) tunica muscularis, (B1) tunica muscularis (outer longitudinal), (B2) tunica muscularis (inner circular), (C) tunica submucosa, (T) tubuloalveolar proventricular glands in the tunica submucosa, (D) tunica mucosa, (E) lamina propria, (P) papillae, (L) lumen, (PD) primary duct, and black arrows are the septa (upper left), secretory cells (upper middle). The gizzard: (A) tunica serosa, (B) tunica muscularis, (B1) inner oblique muscle layer of the tunica muscularis, (B2) circular muscle layer, and (B3) outer longitudinal muscle layer, (C) tunica submucosa, (D) tunica mucosa, (K) koilin, (L) lumen, (LP) lamina propria, and black arrows are the crypts (lower left), and red arrows are the glands (lower middle).

six, 17, and five times, respectively, thicker than in quail. While the current study revealed that the depth of proventricular gland in a mature female Japanese quail is about 1,288.90 µm in diameter and the tunica mucosa is 553.42 µm thick, the proventricular gland height of a 45-day old male Japanese quail is 9.60 µm and the thickness of the mucosal surface is 28.58 µm [23].

Gizzard

The gizzard or the ventriculus is unique in birds among other vertebrates. It is the muscular compartment of the avian stomach and incomparable to the mammalian stomach [9]. It is small and round in shape with tapering ends situated caudal to the proventriculus and relatively located between the lobes of the kidney and partly behind the left lobe of the liver [7,15]. This mechanical stomach is responsible for providing a suitable environment for the physical and chemical reduction of the bird's intricate nutritional diet [9,10,16).

Figure 1 (lower left) shows the different histological parts of the gizzard. The histologic structure of the gizzard wall in quail was composed of four layers or tunics similar to what was found in the proventriculus. These four layers were (innermost to outermost) the tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa. In addition, a keratinized membrane covering called koilin was also present.

The tunica mucosa was the innermost layer consisting of invaginating long mucosal folds embedded in the lamina propria (Figure 1 – lower left and lower middle). The invaginating folds are the so-called gastric pits and at the base of these folds are the crypts [7,19,25]. These gastric pits range up to the glandular layer and subjugate the majority thickness of the mucosa. Simple tubular glands open into the shallow crypts. These straight tubular glands are also bounded by dense connective tissue with collagen and are filled in their upper parts by a material that becomes continuous with the dense layer overlying the mucosa [16]. The gastric glands are also situated in the tunica mucosa and are lined principally by chief cells located in the lower parts of the glands which are cuboidal in shape whose nuclei are more or less rounded in outline. These cells produce a protein-rich secretion [9,16,25]. The basal cells are few as compared to the chief cells. They occur by pair and are located in the depth of the gland. These cells have a large round pale nucleus, complex nucleolus, and pale staining cytoplasm and have no granules. Since they are situated in the mitotic zone of the gland, it could be possible that they are stem cells. There were intermediate cells also found in the deep part of the glands [16]. The gizzard glands are essential in enzyme and cell secretions that are important in the digestion of feeds as well as protection and absorption of nutrients [9].

There were more lamina propria constructed by a loose connective tissue separating the pits between glands. The surface epithelium was composed of simple low columnar cells to simple cuboidal cells with round vesicular basal nuclei. While the epithelium and the lamina propria were both present, there was no muscularis mucosa between the lamina propria and the underlying tunica submucosa (Figure 1 – lower left and lower middle). Previous studies [7,10,12,23,25] also reported the same observations.

The koilin was a thick horny layer of keratinoid that served as a covering membrane of the tunica mucosa (Figure 1 – lower left and lower middle). This layer with a relatively wavy pattern is formed by the secretion of gizzard glands and consists of scaffolding of interrelating vertical rods and horizontal matrix [9,13,14]. The superficial koilin is less dense than the deeper koilin trapped within the glandular epithelium [10]. The sandpaper-like texture of the koilin acts as a grinding surface of the feeds to increase their surface area, to promote better gastric proteolysis, and also to serve as a protection layer [8,16].

The tunica submucosa of the gizzard was indistinguishable from the lamina propria (Figure 1 – lower left). However, it was bulky and composed of abundant loose connective tissue that was richly supplied with blood vessels and nerves as similarly observed in previous studies [7, 21,23].

The tunica muscularis was structured by a well-developed and strongly thick, three smooth muscle layers namely, the outer longitudinal, the middle circular and the inner oblique (Figure 1 – lower left and lower right). This layer comprised the bulk of the organ to justify its major role in grinding and macerating the complex diet of avian species. These results were similar to those of Ahmed et al. [23], Kadhim et al. [10], and Al-Saffar & Al-Samawy [21] but contrary to the findings in coot bird [17] and red-capped cardinal [12]. The muscle bundles are also interposed with bands of connective tissues rich in blood vessels and nervous ganglions of mioenteric or the auerbach plexus [12]. The tunica muscularis is accountable for the strong contractions of gizzard in triturating the ingesta to decrease the size of the feed. It is also responsible for the motility of feeds and back and forth in the proventriculus and for propelling the feeds into the small intestine by muscle contraction [8,16].

The tunica serosa was the outermost layer, and, similar to previous reports [7,19,21], mainly consisting of dense loose connective tissue lined by a simple squamous mesothelium, blood vessels, lymphatic vessels, and nerves. In addition, Catroxo et al. [12] mentioned that it is also constituted by adipose cells and

nervous elements of a plexus.

Table 1 shows the measurements of the different microscopic parts of the quail gizzard. The organ is composed of four main layers, the tunica mucosa, the tunica submucosa, the tunica muscularis which was the thickest, and the tunica serosa which was the thinnest. It can be noted that the thickness of the tunica muscularis decreases as it transitions to the duodenum of the small intestine.

Al-Saffar & Al-Samawy [7] described the mean thickness of the tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa, which was about three, 15, three, and two times, respectively, thicker than the corresponding layer in quail. Moreover, in a study on a 45-day old male Japanese quail [23], the koilin layer was 336.74 µm in thickness which is slightly similar to the measurement obtained in this study in mature female Japanese quail (about 318.36 µm). However, the development of this keratinized layer of the gizzard varies among other avian species. In granivores, insectivores, and herbivores, the gizzard is well-developed and koilin is present while, in carnivores and piscivores, the gizzard is poorly developed and there is an absence of koilin [9,26]. Though koilin was reported in various species of birds [10,17,19,21,23,25], it was not documented as a histologic part in striated scope owl, krestel, and Eurasian sparrowhawk [7].

Based on the findings of the study, the histology of the proventriculus and gizzard of the Japanese quail revealed no remarkable difference compared to the other poultry species, hence, their stomach is almost precisely similar to that of the chicken. However, the histomorphometry of the organs examined had remarkable variations as compared to other avians.

Materials & Methods

Collection of animals and preparation of samples

A total of five mature female Japanese quails were collected from a backyard raiser. The birds were carefully euthanized through cervical dislocation and immediately followed by decapitation [27-29]. The organs were collected and washed using a physiologic saline solution. The tissues were cross-sectionally cut into 1 to 3 cm tissue blocks and preserved in 10 percent buffered formalin. The fixed tissue samples were processed for paraffin technique and stained with H & E stain.

Microscopic examination of tissues

The prepared tissue slides were examined using a compound digital microscope (Olympus CX23, Tokyo, Japan). The major histological parts of the proventriculus such as the layers (tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa), the proventricular glands, and plicae were identified, photographed, and described. Likewise, the microscopic

Table 1.
Histomorphometry of the histological parts of the mature female Japanese quail (*C. coturnix japonica*) proventriculus and gizzard.

Histologic Parts	Proventriculus		Gizzard	
	Thickness (µm)	Diameter (µm)	Thickness (µm)	Depth (µm)
Tunica mucosa	553.42 ± 229.97	-	456.15 ± 104.55	-
Lamina propria	145.58 ± 64.18	-	-	-
Koilin	-	-	318.63 ± 127.66	-
Crypt	-	-	-	226.67 ± 70.87
Tunica submucosa	2,164.37 ± 358.98	-	112.25 ± 69.19	-
Proventricular gland	-	1288.90 ± 402.55	-	-
Papillae	-	431.38 ± 175.74	-	-
Tunica muscularis	235.07 ± 264.63	-	1,480.07 ± 739.43	-
Tunica serosa	22.69 ± 9.11	-	60.44 ± 30.29	-

The data were presented as mean ± standard deviation (SD) of the examined histologic parts (3 to 6 microscope fields of view per section: upper, middle, and lower sections of the proventriculus and gizzard)

structures of the ventriculus such as the layers (the tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa), the glands, and the keratinized layer (koilin) were also identified, photographed, and described. The histomorphometry of these different histological parts was measured using a compound digital microscope (Olympus CX23, Tokyo, Japan) and the images obtained were measured for thickness or diameter in µm using ImageJ software (Java-based, version 1.52v, LOCI University of Wisconsin-Madison) [30].

Statistical analysis

Each organ per bird was measured in triplicate. The measurement data obtained from the histological parts of the randomly selected proventriculus and gizzard tissue sections from the samples were presented as mean ± standard deviation (SD).

Authors' Contributions

F.B.R.M. conceptualized the research, initiated the writing of research outline and the final manuscript, examined the tissues and performed the statistical analyses and interpretation of results, and arranged and reviewed all parts of the manuscript, including formatting. N.A.M.D.C. prepared the samples for tissue processing and staining, examined the tissue slides and performed the image capturing and measurement of histologic parts, and wrote the materials and methods and some parts of the results and discussion. M.B.S.S. examined the captured images before measurement, identified the histologic parts of all tissue samples, and wrote some parts of the results and discussion.

Competing Interests

The authors declare that they have no competing interests.

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Hard ticks circulate *Anaplasma spp.* in South-Khorasan province, Iran

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ABSTRACT

Ticks are vectors for several important zoonoses including different species of *Anaplasma*. The present study aims to determine the presence of *Anaplasma spp.* in hard ticks collected from livestock of South-Khorasan province, Iran. A total of 684 livestock were sampled and 269 ticks were collected. Two genera and 6 species of ticks were identified including *Rhipicephalus sanguineus, Hyalomma detritium, Hyalomma marginatum, Hyalomma anatolicum, Hyalomma asiaticum, Hyalomma dromedarii* and *Hyalomma spp.* Eleven *Hyalomma* nymphs and 3 *Rhipicephalus* nymphs were also identified. 100 Out of 269 ticks were chosen for molecular detection. DNA was extracted followed by PCR technique to detect *Anaplasma spp.* The presence of *Anaplasma spp.* was confirmed in 20 out of 100 tested samples (20%). All positive samples collected from Birjand county were *Rhipicephalus sanguineus*. Results of the present study showed a relatively high infection rate of *Anaplasma* in hard ticks in South-Khorasan Province.

Keywords

Ixodidae, PCR, Hyalomma, Rhipicephalus

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Abbreviations

PCR: Polymerase chain reaction
BLAST: Basic local alignment tool
MSP-4: Major surface protein 4

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Anaplasmosis refers to a disease of animals and humans, caused by obligate intra-erythrocytic bacteria (Family *Anaplasmataceae*: order Rickettsiales), which poses significant livestock economic constraints to countries due to reduction in milk and body weight, abortions, veterinary expenses, and finally animal losses [1]. Species of veterinary interest include *A. bovis, A. centrale, A. marginale, A. ovis, A. phagocytophilum* (common species between humans and animals), and *A. platys*. Clinical evidence of anaplasmosis in livestock is characterized by an acute onset of fever, weight loss, pale or yellowish mucosa, anorexia, milk reduction, and death (if not treated appropriately). After recovery from infection, the animal remains a source of infection forever [2].

Ticks are vectors for several important zoonoses including spotted fever, Rocky Mountains fever, Siberia tick typhus, tularemia, Lyme disease, tick-borne relapsing fever (TBRF), Crimean-Congo hemorrhagic fever (CCHF), babesiosis, and anaplasmosis. Alongside pathogen transmission, tick-induced direct losses to livestock such as bite stress, production loss, physical damage, anemia, and poisoning are also considerable [3]. Ticks are categorized into three families: *Ixodidae, Argasidae*, and *Nuttalliellidae*. The *Ixodidae* family, also called hard ticks, contains most ticks of veterinary importance [4].

According to statistics published by the statistics center of Iran, the population of large and small ruminants in South-Khorasan province is about 140,000 and 34,807, respectively, which play an important role in the economy and life of the people of the region [5]. On the other hand, South Khorasan province shares a 460 km border with Afghanistan which is endemic for many diseases including malaria, leishmaniasis, tick-borne encephalitis, Crimean-Congo hemorrhagic fever, and anaplasmosis. The above facts imply the importance of epidemiological studies on vectors and ruminants in this area [6]. The present study aims to determine the presence of Anaplasma spp. in the hard ticks, collected from livestock in South-Khorasan province, Iran.

Study area and sample collection

Birjand, Qaen, Khusf, Darmian and Sarbisheh counties from South Khorasan province (32.8653°N 59.2164°E) were surveyed. A total of 684 livestock (sheep, goats, cows and camels) were sampled in summer 2019. Multistage random sampling method was used for the collection of tick samples. Hard ticks were randomly collected using forceps and then were placed into labeled tubes. All samples were transferred on ice to the Department of Medical Entomology, School of Public Health, Tehran University of Medical Sciences, Iran for species identification. Ticks were identified at the level of species under stereomicroscope according to valid morphological keys [7].

Molecular detection of Anaplasma

100 out of 269 ticks were chosen for molecular detection according to sex, collection area and tick life stage. Ticks were placed into 70% ethanol for 15 min; air dried and kept in separate tubes. Incubation in liquid nitrogen for 5 min followed by grinding was performed for each tick. DNA extraction was performed by the Exgene extraction kit (GeneAll®, Korea) according to the manufacturer's guidelines. 16s ribosomal RNA gene was targeted to amplify a 524 bp fragment by nested-PCR using two primer sets as follows: Ehr1 5′-GAACGAACGCTGGCGGCAAGC-3′ and Ehr2 5′-AGTA[T/C]CG[A/G]ACCAGATAGCCGC-3′ for the first step and Ehr3 5′-TGCATAGGAATCTACCTAGTAG-3′ and Ehr4 5′-CTAGGAATTCCGCTATCCTCT-3′ for the second step of nested-PCR. Also, an amplicon of 464-bp msp4 was amplified using primers Fmsp4: 5'-GTYARRGGCTAYGRCAAGAG-3' and Rmsp4: 5'-AGTRAACTGGTAGCTWATYCCA-3'. The procedure of nested-PCR was similar to Rar et al. with some modifications in the PCR thermal program [8,9]. 25 µL reaction mixtures contained 2.5 µL PCR buffer, Tris-HCl (10×) (pH 9.0), 0.75 µL MgCl2, 0.5 µL dNTPs, 0.2 µL Taq DNA polymerase, 1 µL of each of forward and reverse primers, and 2 µL of extracted DNA [9]. Confirmed positive DNA samples for *Anaplasma* species were obtained from Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Iran. Double-distilled water was used as the negative control. Products were subsequently analyzed by gel electrophoresis.

Sequencing and phylogenetic Analysis

A pool of 10 positive ticks were sent to Codon Genetic Group® (Tehran, Iran) for sequencing. Sequences were then checked to correct any sources of error using the Chromas® software. The sequence were then blasted by BLAST, National Institute of Health, USA (http://www.ncbi.nlm.nih.gov/BLAST) and submitted to GenBank with accession number [MW428433].

The present sequence was aligned using MEGA7 software (CLUSTALW algorithm) and used in phylogenetic tree construction. Maximum likelihood was used to determine distance among different sequences in the phylogenetic tree.

Statistical analysis

Data were analyzed by SPSS version 19.0. Descriptive statistics were used to summarize the data.

Two genera and 6 species were identified including 111 *Rhipicephalus sanguineus* (41.3%), 24 *Hyalomma detritium* (8.9%), 6 *Hyalomma marginatum* (2.2%), 9 *Hyalomma anatolicum* (3.3%), 5 *Hyalomma asiaticum* (0.9%), 90 *Hyalomma dromedarii* (33.5%) and 10 *Hyalomma spp.* (3.7%). Eleven (4.1%) *Hyalomma* nymphs and 3 (1.1%) *Rhipicephalus* nymphs were also identified. The highest frequency of genus and species was related to the *Hyalomma* and *Rhipicephalus sanguineus,* respectively. The presence of *Anaplasma* was confirmed in 20 out of 100 tested samples (20%). All positive samples collected from Birjand county were *Rhipicephalus sanguineus* (Table 1).

Out of 20 infected ticks, 5 (25%) were isolated from goats and 15(75%) from sheep. The male: female ratio of positive ticks was 12:8. Eighteen infected ticks (90%) were captured from lowland areas while two (10%) were collected from highland areas.

A phylogenetic tree was constructed between the present sequence and registered sequences of MSP-4 in GenBank. Four clades were created for *Anaplasma spp.* including: *A. ovis, A. marginale, A. capra*, and *A. phagocytophilum* (Fig1).

The predominant species in South Khorasan province was *Rhipicephalus sanguineus*. The prevalence of *Anaplasma* infection in hard ticks of the re-

Table 1.
Information related to tick species that were selected for molecular detection.

Species	No. of examined ticks by PCR	Identified specimens (No.)	Identified specimens (%)
Rhipicephalus sanguineus	61	20	32.7
Hyalomma detritium	3	0	0
Hyalomma marginatum	0	0	0
Hyalomma anatolicum	61	0	0
Hyalomma asiaticum	4	0	0
Hyalomma dromedarii	0	0	0
Hyalomma spp.	4	0	0
Total	100	20	20

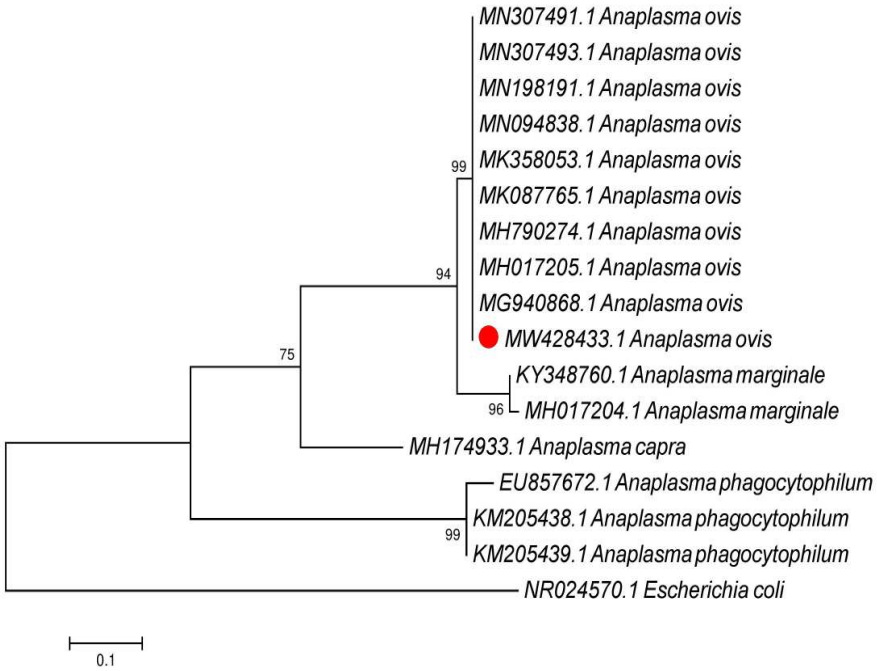

Fig 1.
The evolutionary tree was inferred by using the maximum likelihood method with bootstrap of 1000 replications. The percentage of trees in which the associated taxa clustered together is shown next to the branches. *Escherichia coli* was included as outgroup. The scale bar indicates an evolutionary distance of 0.10 nucleotides per position in the sequence [10,11]. Sequence derived from the present study is marked with a red circle.

gion was 20% which was only related to Birjand county. Infection was found in *Rhipicephalus sanguineus* as the only infected species. Infected ticks were collected from sheep and goats. Ticks collected from other livestock were negative in terms of *Anaplasma* infection. Plains were more infected than highland areas.

In a molecular survey of hard ticks in the Iran-Afghanistan borderline (Sistan region, southeast of Iran), Jafar Bekloo et al. reported that *Anaplasma* was found in 26.4% of tested specimens. The results showed the infection of *Rhipicephalus sanguineus* and *Hyalomma anatolicum* with *Anaplasma ovis* [12]. In a study conducted in the Kerman province (which shares a border with South-Khorasan) rate of tick infection with *Anaplasma* was reported at 23.95% and *Hy. Marginatum* and *Rhipicephalus sanguineus* were infected species [3]. South Khorasan, Kerman, and Sistan regions are neighbors and share common geographical attributes; so they are supposed to show a similar prevalence of tick infection associated with *Anaplasma*. Tick infection with *Anaplasma* in East-Azerbaijan province, southwest of Iran was reported 71% which is higher than east of the country [9]. Jafar Beklooa et al. also reported that tick infection with *Anaplasma* was 25% in the north of Iran and infected species were: *Rh. sanguineus*, *Rh. bursa*, *Hy. Marginatum* and *Hy. Scupense*, a finding which was very different from the present study in terms of infected tick species [13]. A gene fragment of *Anaplasma* species was identified in 49.5% and 59% of tested ticks in the Mazandaran and Savadkouh regions of Iran, respectively [1,14]. From the previous studies, it can be concluded that although the prevalence of tick infection with *Anaplasma* is variable in different parts of Iran, the most prevalent tick genus with *Anaplasma* infection is *Rhipicephalus*.

Results of the present study showed a relatively high infection rate in hard ticks collected from livestock in South-Khorasan Province. It seems that controlling and preventive policies related to the spread of anaplasmosis in South Khorasan province should be pursued more seriously. In addition, due to the common border with Afghanistan, there is a possibility of cross-border transmission. More studies in terms of tick infection with other arthropod-borne pathogens are also recommended in South-Khorasan.

Authors' Contributions

A.J., S.A., M.R. and A.H. conceived and planned the experiments. A.J., S.A., F.F. and M.F. carried out the experiments. A.J., D.S., A.L, M.B. and S.A. contributed to sample preparation. A.J., D.S., M.R., M.B. and A.L. contributed to the interpretation of the results. A.J. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Abstracts (in Persian)

دامپزشکی و حرفه پرورش دام‌های مولد غذا در عصرِ ردّپاها و سلامت یک‌پارچه: راهبردی توصیفی

کامران شریفی

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چکیده

جهان فعلی که دنیایی است پویا و دردگرگونی دائمی، ضرورتاً نیازهای جدیدی را در سطح جامعه ایجاد کرده تاثیرات ژرفی بر اکوسیستم‌ها، فرهنگ‌ها و حرفه‌ها نهاده است. بی‌توجهی به نیازها و پی‌آمدهای محیطی مرتب در حال تغییر، ممکن است اثرات مخربی برای تمامی جنبه‌های حرفه دامپزشکی داشته باشد. با افزایش ارتباطات جهانی، چند مفهوم خلق شده است که حرفه دامپزشکی باید آن‌ها را در مشی خود لحاظ نماید، در غیر این صورت، بروز برخی ناپایداری‌ها هم در حرفه و هم در جامعه غیر قابل اجتناب خواهد بود. در این نوشتار، این مفاهیم جدید نقادانه تحلیل خواهد شد و یک سیمای ترکیبی با دورنمایی یکپارچه ارائه خواهد شد تا به ضرورت‌های لازم برای نیل به موفقیت اقتصادیِ حرفه‌های فعال در حوزه پرورش دام‌های مولد غذا و همچنین نقش پیچیده‌ای که دامپزشکی باید در آینده برعهده بگیرد، اشاره خواهد شد. مفهوم اول، آموزش دامپزشکی متحول است که سازمان جهانی بهداشت دام در ۲۰۰۹ آن را ارائه نمود تا مشخصات لازم برای یک دامپزشکِ با صلاحیت را توصیف نماید، مشخصاتی که دامپزشک نوین را قادر می‌سازد تا به نیازهای داد و ستد و تجارت مدرن پاسخ دهد و خود را با آن منطبق کند. مفهوم دوم سلامت یکپارچه است که دورنمایی از یک بهداشت یکپارچه و همه شمول را ارائه می‌کند، به نوعی که اگر حروف کلمه سلامت Health را به عنوان "سرنام" در نظر بگیریم، H نشانه انسان، E نشانه اکوسیستم، A نشانه حیوانات، L و T به معنی زیستن با هم دیگر، و H آخر به معنی در"در هماهنگی" است. سومین مورد، "تئوری آب مجازی" است که کل آب مصرفی در روند هر فعالیت را با مفهوم ردّ پای آب اندازه می‌گیرد. مطابق این تئوری، برای تولید هر کیلوگرم شیر و گوشت، به ترتیب ۱،۰۰۰ و ۱۵،۵۰۰ لیتر آب مصرف می‌شود. مفهوم آخر، "ردّپای کربنی" است که میزان ورود گازهای گلخانه‌ای به جو را بر اساس معادلِ دی اکسید کربنی که از طریق فعالیت‌های انفرادی، رویدادها، سازمان‌ها، خدمات، مکان‌ها، محصولات یا صنایع وارد اتمسفر می‌شود را بیان می‌کند. نقش خطیر حرفه دامپزشکی و مسئولیت آن در یکپارچه نمودن این چهار مفهوم، موضوع بحث در این مقاله است.

واژگان کلیدی

ردّپای آب، ردّپای کربن، سلامت یکپارچه، حیوانات مولد غذا، پایداری محیط زیست

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شناسایی ویروسهای جهش یافته برونشیت عفونی لینیج GI-23 از گله های مرغ تجاری در استان خراسان رضوی، ایران در سال ۲۰۱۹

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۲ اداره کل دامپزشکی استان خراسان رضوی، مشهد، ایران.

چکیده

کرونا ویروسی با نام ویروس برونشیت عفونی (IBV) ایجاد کننده برونشیت عفونی (IB) به عنوان یکی از مهمترین بیماریهای تنفسی در طیور می باشد. اجرای اقدامات پیشگیرانه از جمله واکسیناسیون و امنیت زیستی برای کنترل بیماری ضروری است. شناسایی مسیر ورود ویروس های جدید به یک منطقه برای حفظ امنیت زیستی مهم است و مشخص کردن مارکرهایی مانند جهش های منحصر به فرد، ویروس ها را قابل ردیابی می کند. در مطالعه تعیین ژنوتیپ در استان خراسان رضوی برای گله های مرغ تجاری آلوده به IBV تعداد ۱۱ ویروس از ۱۱ گله در شهرهای مختلف با روش مولکولی PCR شناسایی شد. تعیین توالی ناحیه متغیر ژن S1 و به دنبال آنالیز فیلوژنتیک نشان داد که هشت ویروس در لینی(Is-Variant2) GI-23 ، دو ویروس در لینیج(Mass) GI-1 و یک ویروس در لینیج (793B) GI-12 طبقه بندی می شوند. اگرچه ویروسهای شناسایی شده لینیج GI-23 از ایران نشأت گرفته اند، هفت ویروس دارای جهش های مترادف (T954C و G1056A) و غیر مترادف (C797T) هستند که قبلاً گزارش نشده است. نتایج این مطالعه نشانگر وقوع تغییرات ژنتیکی جدید در IBV های ایرانی از لینیج GI-23 در دو منطقه مختلف در استان خراسان رضوی است. در برنامه های نظارتی آینده، ردیابی شیوع این ویروس ها و ارزیابی اجرای برنامه های امنیت زیستی امکان پذیر خواهد بود. این مطالعه نشان می دهد که شیوع بالای ویروس های لینیج GI-23 در ایران ممکن است احتمال جهش ویروس را افزایش دهد، بنابراین ممکن است سویه های ویروسی با بیماری زایی متفاوت ایجاد شود.

واژگان کلیدی

IBV، تعیین ژنوتایپ، لینیج GI-23، جهش ژنتیکی، ایران

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نقش میانجیگری مسیر نیتررژیک مرکزی بر هیپوفاژی القایی توسط اکسی توسین در جوجه های تخمگذار

مرتضی زنده دل، مینا خدادادی، حمیرا زندیه، کسری مختارپوریانی، بهروز رحمانی، علی باغبان زاده

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چکیده

سیستم های نوروترانسمیتری متنوعی در تنظیم اخذ غذا در پستانداران و پرندگان نقش دارند. اکسی توسین و نیتریک اسید بعنوان عوامل هیپوفاژیک در پرندگان شناخته شده اند. فرضیه ما احتمال وجود یک تداخل بین سیستم نیتررژیک و اکسی توسین در تنظیم اخذ غذا در جوجه های تخمگذار نوزاد بود. در تمامی آزمون ها جوجه های سه ساعت محروم از غذا، محلول کنترل یا داروها را به روش تزریق داخل بطن مغزی دریافت کردند. سپس بصورت آزاد به غذا و آب دسترسی داشتند و اخذ غذای تجمعی (برحسب گرم) در آن ها بر اساس درصدی از وزن بدن اندازه گیری شد. نتایج نشان داد که تزریق داخل بطن مغزی ال-آرژینین (۲۰۰نانومول) هیپوفاژی القایی توسط اکسی توسین (۱۰ میکروگرم) را تقویت کرد و تزریق همزمان ال-آرژینین (۲۰۰نانومول) و اکسی توسین (۲.۵ یا ۱۰میکروگرم) اخذ غذا را بطور معنی داری کاهش داد. در نتیجه نیتریک اکساید احتمالاً کاهش اخذ غذای ناشی از اکسی توسین را در جوجه های نوزاد تخمگذار میانجی گری می کند.

واژگان کلیدی

ال-نیم، ال-آرژینین، اخذ غذا، پرنده

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نقشی برای مسیر گابائرژیک در کنترل تولیدمثل موش های صحرایی ماده از طریق تنظیم بیان ژن های گرلین، کیس پپتین و RFRP-3

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چکیده

کیس پپتین آزادسازی هورمون آزادکننده گنادوتروپین(GnRH) را تحریک می کند. گرلین،پپتید وابسته به آرژنین فنیل آلانین آمید (RFRP-3) و گاما آمینوبوتیریک اسید(GABA) ورودی مهاری به نورون های GnRH ارسال می کند. سندروم تخمدان پلی کیستیک (PCOS) با کاهش سطوح گرلین وRFRP-3و افزایش GnRH/LH و کیس پپتین همراه است. در تحقیق حاضر، اثرات باکلوفن (آگونیست گیرنده ی GABAB) بر بیان ژن‌های RFRP3، KiSS1، GnRH و گرلین در هیپوتالاموس موش‌های صحرایی PCOS بررسی شد. برای القای PCOS، موش صحرایی ماده نژاد ویستار به وزن ۲۰۰-۱۸۰g تزریق داخل عضلانی استرادیول والرات را دریافت کردند. سپس، ۱۵ موش صحرایی PCOS در سه گروه تزریق داخل صفاقی سالین یا باکلوفن را با مقادیر ۵ یا ۱۰ mg/kg به مدت دو هفته دریافت کردند. پنج موش صحرایی سالم به عنوان گروه کنترل سالم سالین را دریافت کردند. یک روز بعد از آخرین تزریق، نمونه‌های هیپوتالاموس جداسازی شدند و میانگین بیان نسبی ژن‌های RFRP3 ، KiSS1، GnRH و گرلین با روش واکنش زنجیره ای پلیمراز ریل تایم (RT- PCR) اندازه‌گیری شد. باکلوفن سبب کاهش معنی دار میانگین بیان نسبی ژن های GnRH، KiSS1 در مقایسه با گروه PCOS شد. در حالی که در موش های صحرایی دریافت کننده باکلوفن، میانگین بیان نسبی ژن های RFRP3 و گرلین در مقایسه با گروه PCOS افزایش معنی داری پیدا کرد. مسیر پیام رسانی GABAergic ممکن است فعالیت نورونیGnRH را از طریق تنظیم کاهشی یا افزایشی نوروپپتیدهای داخل هیپوتالاموسی در بالادست نورون های GnRH مهار نماید.

واژگان کلیدی

باکلوفن، GnRH، کیس پپتین، گرلین، RFRP-3.

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ارزیابی درمان های هورمونی برای سناریوهای مختلف کیست های تخمدانی فولیکولی در گاوهای شیری

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چکیده

هدف از مطالعه حاضر، ارزیابی کارآیی تداخلات هورمونی مختلف در درمان کیست های تخمدانی فولیکولی (COF) بر اساس سناریوهای مختلف شامل اندازه کیست و حضور سایر فولیکول ها بر روی تخمدان های گاوهای شیری است. در مجموع ۱۹۹ گاو هلشتاین مبتلا به COF در طی ۱۰۰ روز اول پس از زایش وارد مطالعه شدند. این گاوها به صورت تصادفی در ۴ گروه تقسیم شدند:۱) گروه GnRH (G : تزریق داخل عضلانی ۱۰۰ µg گنادورلین استات در روز صفر و ۱۵۰ µg دی-کلوپروستنول ۷-۱۲ روز بعد، ۲) گروه ۲ :Double GnRH (DG) دو تزریق داخل عضلانی ۱۰۰ µg گنادورلین استات در فواصل ۶ ساعته در روز صفر و دی-کلوپروستنول ۷-۱۲ روز بعد، ۳) گروه وسیله داخل مهبلی حاوی پروژسترون (IPD): جاگذاری PRID-Delta برای ۷-۱۲ روز و تزریق دی-کلوپروستنول در روز خارج کردن وسیله، ۴) گروه کنترل: تزریق داخل عضلانی ۲ ml سالین استریل در روز صفر و ۷-۱۲ روز بعد. میزان درمان COF به شکل معنی داری در گروه های G و DG در مقایسه با گروه های IPD و کنترل بهبود یافت. هیچ اختلاف معنی داری بین گاوها در گروه های G و DG وجود نداشت. در گروه کنترل، گاوهایی با کیست تخمدانی کوچکتر از ۲/۵ سانتی متر در مقایسه با سایر گاوها به شکل معنی داری بهبود خود به خودی بهتری داشتند. در نتیجه، این مطالعه، بهبود بالینی خوبی را در گروه هایی از گاوها که با GnRH درمان شدند، نشان داد. با این حال، هیچ بهبودی در عملکرد تولیدمثلی این دام ها مشاهده نشد.

واژگان کلیدی

گاو شیری، GnRH، پروژسترون، کیست تخمدانی فولیکولی

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شناسایی مولکولی و آنالیز فیلوژنتیک کلامیدوفیلا آبورتوس جداشده از گوسفند و بز

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چکیده

کلامیدیوفیلا آبورتوس یکی از مهمترین عوامل ضعف آبستنی (سقط جنین) در گوسفند و بز در بیشتر کشورها است. در مطالعه حاضر نمونه های شیر گله های گوسفند و بز استان آذربایجان غربی برای تشخیص کلامیدیوفیلا آبورتوس با استفاده از روش واکنش زنجیره ای پلی مراز و توالی یابی نوکلئوتیدی مورد آزمایش قرار گرفت. تعداد ۳۶۰ نمونه شیر بطور تصادفی از میش ها (تعداد ۱۸۰ نمونه) و بزها (تعداد ۱۸۰ نمونه) متعلق به ۱۸ گله از مناطق مختلف استان آذربایجان غربی در طول سال ۱۳۹۷ اخذ گردید. اسید نوکلئیک نمونه های شیر جدا سازی شدند و روش واکنش زنجیره ای پلی مراز آشیانه ای برای ژن هدف 16S rRNA جهت شناسایی گونه های کلامیدیا مورد استفاده قرار گرفت. ژنomp (پروتئین غشای بیرونی) آن جهت توصیف کلامیدیوفیلا آبورتوس تکثیر گردید و توالی یابی نوکلئوتید های آن انجام شد. نتایج نشان دادکه ۸.۶۱ درصد (CI: ۶.۱۳٪–۱۱.۹۶٪ ۹۵٪) کل نمونه های مورد آزمایش از نظر کلامیدیوفیلا آبورتوس مثبت بودند. (۱۱.۶۷ درصد شیر گوسفند و ۵.۵ درصد شیر بزان). ۵۰ درصد گله های مورد ازمایش از نظر الودگی به کلامیدوفیلا ابورتوس مثبت بودند. میزان نمونه های مثبت در منطقه مرکزی استان بطور معنی داری از سایر مناطق بالاتر بود(p <۰.۰۵). نمونه های مثبت کلامیدیایی در حیواناتی که سابقه سقط جنین داشتند بطور معنی دار بالاتر از مواردی بود که بدون سابقه سقط جنین بودند(p <۰.۰۵). نمونه های مثبت در فصل پاییز بطور معنی دار نسبت به سایر فصول بالاتر بود(p < ۰.۰۵). وهمچنین شیر حیواناتی که در گروه سنی بالای ۴ سال بودند بطور معنی داری از سایر گروه های سنی موارد مثبت بالاتری داشتند(p < ۰.۰۵). انالیز فیلوژنتیکی بر اساس ژن هلیکاز نشان داد که دو جدایه توالی یابی شده با سایر جدایه های گزارش شده در بانک ژنی قرابت نزدیک داشتند. نتیجه گیری اینکه نشخوارکنندگان کوچک در استان آذربایجان غربی با کلامیدیوفیلا آبورتوس الوده شده اند و آنها قادر به انتقال و ترشح این باکتری به شیر می باشند و این مسئله هشدار جدی برای انتقال این ارگانیسم از شیر و محصولات لبنی به انسان می تواند باشد.

واژگان کلیدی

شیر، کلامیدیوفیلا آبورتوس، ژن omp، واکنش زنجیره ای پلی مراز آشیانه ای، ژن هلیکاز

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افزایش خصوصیات تعدیل ایمنی سلول‌های بنیادی مزانشیمی با پیش‌تیمار ویتامین E و hCG

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۲ گروه زیست‌شناسی، دانشکده علوم، دانشگاه فردوسی مشهد، مشهد، ایران.
۳ گروه جراحی، دانشکده پزشکی، دانشگاه آزاداسلامی مشهد، مشهد، ایران.
۴ گروه زیست‌شناسی، دانشکده علوم، دانشگاه حکیم سبزواری، سبزوار، ایران.

چکیده

سلول های بنیادی مزانشیمی برای درمان اختلالات مختلف ایمنی به کار رفته‌اند. کشت طولانی مدت این سلول‌ها در شرایط برون‌تنی باعث کاهش کارکرد درمانی آنها می‌شود. پیش تیمار سلول‌های بنیادی مزانشیمی با برخی مواد شیمیایی در محیط آزمایشگاهی می تواند این محدودیت را تاحدی برطرف نماید. در این مطالعه سلول‌های بنیادی مزانشیمی مشتق از بافت چربی با hCG که یک هورمون گلیکوپروتئینی است و ویتامین E که آنتی اکسیدانت است، پیش تیمار شدند و خصوصیت تعدیل ایمنی این سلول ها پس از پیش تیمار بررسی شد. برای این منظور، برای جداسازی سلول‌های بنیادی مزانشیمی مشتق از چربی از ضایعات عمل لیپوساکشن استفاده شد. ابتدا سلول‌ها با با لیپوپلی‌ساکارید القاء شدند. سپس با ۱۰ واحد بین‌المللی hCG و ۶۰۰ میکرومول از ویتامین E برای مدت ۲۴ ساعت تیمار شدند. بیان ژن‌های IL-1β، COX-2، TSG-6 و IL-6 در سطح mRNA در دو گروه تیمارشده و کنترل بررسی شد. برای ارزیابی عمکرد سلو ل‌های تیمارشده، هم‌کشتی با سلول های تک‌هسته‌ای خون محیطی (PBMC) انجام شد. نتایج نشان داد که پیش تیمار با hCG و ویتامین E موجب بیان کمتر ژن‌های پیش‌التهابی IL-1β، COX-2 و IL-6 شد. درحالیکه بیان TSG-6 افزایش معنی‌داری نداشت. همچنین هم‌کشتی سلول‌های دو گروه با PBMC نشان داد میزان PBMC در گروه تیمارشده به شکل معنی‌داری کمتر است. این نتایج نشان داد پیش تیمار سلول‌های بنیادی مزانشیمی مشتق از بافت چربی با hCG و ویتامین E می‌تواند باعث افزایش خصوصیت تعدیل ایمنی این سلو ل‌ها شود.

واژگان کلیدی

تعدیل ایمنی، سلول‌های بنیادی مزانشیمی، پیش تیمار، ویتامین E ، hCG

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اثر اسانس مریم‌گلی و مرزه بر باکتری‌های اصلی ورم پستان گاو

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چکیده

درمان بیماریهای باکتریایی با آنتی بیوتیک‌ها مشکلاتی نظیر مقاومت دارویی و باقیمانده دارو در محصولات دامی دارد. اسانس گیاهان دارویی فعالیت ضدباکتریایی داشته و از بهترین جایگزین‌ها هستند. این مطالعه به‌منظور بررسی فعالیت ضدباکتریایی اسانس‌های مرزه و مریم‌گلی علیه باکتری‌های اصلی ورم پستان گاو شامل استافیلوکوک آرئوس، استرپتوکوک آگالاکتیه و اشرشیاکلی انجام شد. روش گازکروماتوگرافی متصل به طیف نگار جرمی برای شناسایی ترکیبات شیمیایی اسانس استفاده شد. روش رقیق‌سازی متوالی با استفاده از شیر بجای محیط کشت سنتتیک برای تعیین حداقل غلظت مهاری و حداقل غلظت کشندگی اسانس‌ها انجام گردید و تاثیر اسانس‌ها بر منحنی رشد باکتریها در محیط شیر در ساعت‌های ۰، ۱، ۲، ۴، ۱۰و ۲۴ مطالعه شد. مهمترین ترکیبات مریم‌گلی کارواکرول (۶۱/۰۱ درصد)، تیمول (۲۰/۴۱ درصد)، یک- آر- آلفا- پینن (۷/۸۸ درصد) و مرزه اکالیپتول(۳۲/۴۵ درصد)، تیمول(۲۸/۲۴ درصد) و آلفا- پینن (۱۳/۴۲ درصد) بود. محدوده حداقل غلظت مهاری و کشندگی مرزه ۱/۲۵-۲/۵ و ۲/۵-۵ درصد و مریم گلی ۱/۲۵- ۰/۶۲۵ و ۲/۵-۱/۲۵ درصد بود و غلظت تحت حداقل غلظت مهاری اسانس‌ها جمعیت باکتری اشرشیاکلی و استافیلوکوک آرئوس را در ساعت‌های ۴، ۱۰ و ۲۴ و جمعیت اشرشیاکلی را در ساعت‌های ۱۰ و ۲۴ بطور معنی‌داری کاهش دادند. اسانس مرزه و مریم‌گلی اثرات ضد باکتریایی علیه هر سه باکتری داشتند و مریم‌گلی با دارا بودن ترکیبات ضدباکتریایی قوی (کارواکرول و تیمول) بیشتر، اثر قوی‌تری از مرزه علیه باکتریها داشت و مطالعات بالینی برای اثر درمانی اسانس مریم گلی در بیماری ورم پستان توصیه می‌شود.

واژگان کلیدی

آنتی بیوتیک، گیاهان دارویی، مریم گلی، مرزه

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کنه های سخت، چرخه ی گونه های آناپلاسما را در استان خراسان جنوبی، ایران حفظ می کنند

امیرسجاد جعفری[۱]، سحر اسدالهی زوج[۱]، مهدی راسخ[۲]، داریوش سعادتی[۳]، فائزه فقیهی[۴]، مهدی فضلعلی پور[۵]، ابوالفضل لطفی[۶]، مجتبی بیک محمدی [۷]، اسداله حسینی چگنی*[۸]

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چکیده

کنه ها ناقل چندین بیماری مشترک بین انسان و دام هستند که شامل گونه های مختلف آناپلاسما می شود. هدف از مطالعه حاضر تعیین حضور گونه های مختلف آناپلاسما در کنه های سخت جدا شده از دام های اهلی استان خراسان جنوبی، ایران است. ۶۸۴ دام اهلی بررسی شدند و ۲۶۹ کنه سخت از آنها صید شد. دو جنس و شش گونه کنه ای شامل ریپیسفالوس سانگوئینوس، هیالوما دتریتیوم، هیالوما مارژیناتوم، هیالوما آناتولیکوم، هیالوما آسیاتیکوم، هیالوما درومداری وگونه های هیالوما شناسایی شدند. ۱۱ نمف هیالوما و ۳ نمف ریپیسفالوس نیز شناسایی شدند. ۱۰۰ کنه برای آزمایشات مولکولی انتخاب شدند. پس از استخراج ماده ژنتیک، واکنش زنجیره ای پلیمراز برای شناسایی گونه های آناپلاسما انجام شد. وجود آناپلاسما در ۲۰ نمونه از ۱۰۰ نمونه کنه آزمایش شده (۲۰٪) تأیید شد. تمام نمونه های مثبت متعلق به جنس کنه ی ریپیسفالوس سانگوئینوس و از شهرستان بیرجند جمع آوری شده بود. نتایج مطالعه حاضر میزان آلودگی نسبتاً بالایی از آناپلاسما را در کنه های سخت در استان خراسان جنوبی نشان داد

واژگان کلیدی

کنه ی سخت، واکنش زنجیره ای پلیمراز، هیالوما، ریپیسفالوس

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Gene names: The standard gene names, as provided by HGNC (HUGO Gene Nomenclature Committee) should be used. Gene names must be italicized. If the case of mammalian species and if gene names refer to rodent species, they must be upper case; if they refer to non-rodent species they must be written in capitals. If they refer to other species, they must written lower case. Protein names are written in capitals and are not italicized. As an example:

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Quantitative PCR: If the quantitative PCR method has been used, the related section in Materials and Methods must be written following the reference:

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22.

The following information must be provided in the section:

Protocol for DNA/RNA extraction, including quantification and determination of purity;

Reverse transcription (if used): amount of RNA, concentration of all reagents: primers concentration (either random primers or oligonucleotides), reverse transcriptase and master mix components;

qPCR: sequence of forward and reverse primers, probes, amplicon size, accession number of Genebank; thermocycler parameters (i.e. denaturation, annealing and extension steps, number of cycles, melting curves); validation of PCR products; non-template controls for reverse transcription and qPCR should be included in all reactions; and

Data analysis: details for the quantitative or relative analysis.

Use of antibodies: Authors must show that the antibodies are validated and their specificity sis confirmed.

References

Must be up-to-dated and limited to those that are necessary. Lists of references should be given in numerical order in the text, and in the reference list. Please use Vancouver style. To download the Vancouver Style follow the link in the IJVST website which could be used in the Endnote software.

Example piece of text and reference list :

An unhealthy diet, obesity and physical inactivity play a role in the onset of type 2 diabetes, but it has been shown that increased physical activity substantially reduces the risk [1], and participation in regular physical activity is one of the major recommendation of the evidence based guidelines for the primary prevention of diseases [2]. According to the 2004-05 National Health Survey, more than half a million Australians (3.5% of the population) have diabetes mellitus which had been medically diagnosed and most of these people have the Type 2 condition [3]. Gestational diabetes is also on the increase, rising steadily between 2000-01 and 2005-06 [4]. Approximately two thirds of those with diabetes have been prescribed medication [3], but it is of concern that a recent review of the literature found that many people do not take their medication as prescribed [5]. Many patients also self monitor the disease by measuring their blood glucose levels with a glucose meter but Song and Lipman [6] have concerns about how well this is managed.

References for the above example:

1. Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015.

2. Eckerman AK, Dowd T, Chong E, Nixon L, Gray R, Johnson S. Binan goonj: bridging cultures in Aboriginal health. 3rd ed. Chatswood, NSW: Elsevier Australia; 2010.

3. Johnson C, Anderson SR, Dallimore J, Winser S, Warrell D, Imray C, et al. Oxford handbook of expedition and wilderness medicine. Oxford: Oxford University Press; 2015.

4. McLatchie GR, Borley NR, Chikwe J, editors. Oxford handbook of clinical surgery. Oxford: Oxford University Press; 2013.

5. Petitti DB, Crooks VC, Buckwalter JG, Chiu V. Blood pressure levels before dementia. Arch Neurol. 2005 Jan;62(1):112-6.

6. Liaw S, Hasan I, Wade, V, Canalese R, Kelaher M, Lau P, et al. Improving cultural respect to improve Aboriginal health in general practice: a multi-perspective pragmatic study. Aust Fam Physician. 2015;44(6):387-92.

Tables

Please submit tables as individual files and editable text and not as images. Place all table notes below the table body. Each table should have a title which is followed by explanation of results shown in the table. Use of vertical rules must be avoided. Tables should be self-explanatory, and clearly arranged. Tables should provide easier understanding and not duplicate information already included in the text or figures. Each table should be typewritten with double spacing on a separate file and numbered in order of citation in the text with Arabic numerals. Each table should have a concise heading that makes it comprehensible without reference to the text of the article. Explain any non-standard abbreviations in a footnote to the table.

Figures

Figures must be submitted in individual files (format: TIFF, Dimensions: Width: 789 – 2250 pixels at 300 dpi Height maximum: 2625 pixels at 300 dpi, Resolution: 300 – 600 dpi, file size: less than 10 MB, Text within figures: Arial or Symbol font only in 8-12 point). The text and other labels should be placed in the figure as un-compressed layers. Each figure should have a title which is followed by explanation of results shown in the figure. Figures should be numbered in order of citation in the text with Arabic numerals.

For the use of bar diagrams the following publication should be consulted:

Weissgerber TL, Milic NM, Winham SJ, Garovic VD. Beyond bar and line graphs: time for a new data presentation paradigm. PLoS Biol. 2015 Apr 22;13(4):e1002128.

The bar diagrams should be provided in color and in a well-designed and professional format. Please do not use different shades of gray. The axes of diagrams should have titles and units. Also, the source file of the image (Excel etc.) should be provided for typesetting.

Illustrations should be numbered as cited in the sequential order in the text, with a legend at the end of the manuscript. Color photographs are accepted at no extra charge. The editors and publisher reserve the right to reject illustrations or figures based upon poor quality of submitted materials.

If a published figure is used, the publisher's permission needs to be presented to the office, and the figure should be referenced in its legend.

Use of Italics

Gene symbols, Latin terms (i.e. *in vivo, in vitro, ex vivo, in utero, in situ*, and etc.) and species scientific names (using the binomial nomenclature), should be typed in italics, while the first letter of the genus name must be capitalized (i.e. *Homo sapiens*).

Publication Ethics

PUBLICATION ETHICS

Iranian Journal of Veterinary Science and Technology is aligned with COPE's (Committee on Publication Ethics) best practice guidelines for dealing with ethical issues in journal publishing and adopts the COPE guidelines. The journal members (editor, editorial board and the journal manager) have agreed to meet the purposes and objectives of the Journal.

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IJVST requires authors to confirm that they and their co-authors meet all four criteria for authorship based on the guidelines of The International Committee of Medical Journal Editors (ICMJE) (verbatim as follows):

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2. Drafting the work or revising it critically for important intellectual content; AND
3. Final approval of the version to be published; AND
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

The section "Author Contributions" in the manuscript should illustrate and clarify who contributed to the work and how. If a contributor does not meet all four above criteria should be acknowledged in the "Acknowledgements" section of the article.

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Written authorization from all authors for publication of the article is mandatory for IJVST to start the review process. This form entitled "Conflict of interest declaration and author agreement form" must be signed and completed by all authors. This statement and signatures certifies that all authors have seen and approved the manuscript being submitted. Also, the authors by signing this form warrant that the article is the Authors' original work, that the article has not received prior publication and is not under consideration for publication elsewhere, and that the corresponding author shall bear full responsibility for the submission.

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Ethical guidelines for Peer reviewers

Iranian Journal of Veterinary Science and Technology (IJVST) follows and adheres to COPE Ethical Guidelines for Peer Reviewers. IJVST peer reviews all submitted manuscripts with contents in the scope of the journal. The process has been explained in the section "Peer Review Process".

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Peer Review Process

PEER REVIEW PROCESS

Iranian Journal of Veterinary Science and Technology peer reviews all submitted manuscripts with contents within the scope of the journal.

Initial assessment

The submitted manuscript will be subjected to a primary review by the editor or a member of the editorial board for suitability and relevance of the findings to the scope of the journal and quality of the science presented in the paper (sufficient originality, having a message that is important to the general field of Veterinary Medicine, quality of data, novelty, English language, and overall manuscript quality) within two weeks. If the paper is evaluated to be relevant to the scope of the journal and having enough scientific rigor and novelty, it will be sent for the next stage. Otherwise, those manuscripts which are evaluated as not-appropriate in the initial review will be rejected at this stage.

Initial screen

The initial screen will be performed by the editorial office for the structure and format of the manuscript.

Peer review (double-blind)

The manuscripts which are found to be appropriate after the initial screen will be sent for external review by experts in the related field. We have prepared a checklist for reviewers that summarizes their evaluation of the manuscript. The items in this checklist are:

1. TITLE is clear and adequate
2. ABSTRACT clearly presents objects, methods, and results.
3. INTRODUCTION well-structured and provides a rationale for the experiments described.
4. MATERIALS AND METHODS are sufficiently explained and is detailed enough to be reproduced.
5. RESULTS are clearly presented and supported by figures and tables.
6. DISCUSSION properly interprets the results and places the results into a larger research context, and contains all important references.
7. Conclusions are logically derived from the data presented.
8. English Language/style/grammar is clear, correct, and unambiguous.
9. Figures and tables are of good quality and well-designed and clearly illustrate the results of the study.
10. References are appropriate.
11. Regarding this article are you concerned about any issues relating to author misconduct such as plagiarism and unethical behavior.
12. Comments on the importance of the article.

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Based on the reviewers' recommendations a final decision is made by the editor and if needed the help of a member of the editorial board (depending on the field of study). Decisions will include accept, minor revision, major revision with and without re-review, and reject. We aim to reach a final decision on each manuscript as soon as their review results are available.

Author Index

Author index

Author Index

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