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Can qPCR on pooled sheep milk detect brucellosis as part of herd brucellosis control and eradication programs?

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Abstract

Background Brucellosis is a zoonotic disease occurring worldwide. *Brucella melitensis* is the main cause of malta fever in humans and a major cause of abortion in sheep. In herd control and prevention programs, applying a suitable noninvasive method for accurate, rapid, and cost-effective monitoring of infected animals is a major concern. This study aimed to investigate lots of pooled sheep milk using qPCR to detect *Brucella* spp. infection in sheep. By calculating the limit of detection of *Brucella* in qPCR and microbial culture, the maximum number of pooled milk lots that retained the ability to be identified positively for *Brucella* was determined. A total of 144 milk samples were collected from the different seropositive sheep herds. The samples were randomly divided into six groups, each further divided into two subgroups, respectively. Then, DNA extraction was performed on 186 pooled and individual samples, followed by qPCR.

Results The minimum detectable limits for qPCR and microbial culture per ml of milk were 100 and 300 CFU, respectively. Only 40% of the samples in microbial culture tested positive when the concentration decreased to 200 CFU. The results of qPCR indicated that four pools of 24 tested positive, whereas two pools tested negative. After examining the subgroups and individual samples within the two negative groups, it was revealed that all qPCR tests for these samples were negative. In the positive pools, at least one of the samples in the subgroups and corresponding individual samples tested positive. The two positive pools of 24, contained only one individual positive sample in each, indicating that the qPCR test could detect a positive *Brucella* sample in a pool of 24.

Conclusions This noninvasive (milk instead of blood), rapid, and cost-effective method can be used to monitor suspected herds to identify infected animals with fewer tests.

Keywords *Brucella*, Sheep, Pooled milk, QPCR

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Background

According to WHO, brucellosis is a common disease affecting humans and animals worldwide, with over 500,000 new human cases annually [1]. Brucellosis is transmitted to humans through direct contact with animal tissues (placenta, fetus, uterine secretions, etc.) or indirectly by consuming unpasteurized milk and dairy products contaminated with bacteria [2–4]. Three *Brucella* species are commonly associated with human brucellosis: *Brucella melitensis*, *Brucella abortus*, and *Brucella suis* [5]. *Brucella melitensis*, the causative agent of brucellosis in sheep, has been identified as the primary pathogen associated with the global spread of human brucellosis because its infective dose is 10,000 times lower than that of *Brucella abortus* [6, 7]. Unlike cow milk, sheep milk is commonly used to produce traditional unpasteurized dairy products, which increases the likelihood of brucellosis transmission to consumers and causes milk-related Malta fever [8]. In developing country due to traditional and cultural background, the raw milk and unpasteurized dairy products from different animals (cows, goats, sheep, donkeys, buffaloes, yaks, and camels) have gained widespread acceptance as they are more affordable and accessible [9]. In the same way, In Iran the significant risk factors for human infection are related to the consumption of unpasteurized dairy products [10]. Clinical manifestations of brucellosis in sheep include abortion, stillbirth, and reproductive disorders. Brucellosis is a notifiable disease in most countries, and its impact on the livestock industry and public health is significant. Control measures are based on prevention and eradication strategies [11] which highlights the importance of using highly sensitive and rapid diagnostic method. Currently, diagnosing Brucellosis in sheep involves isolating *Brucella* from milk samples or detecting specific antibodies in serum [12]. The gold standard for diagnosing Brucella infection is bacterial isolation using microbial cultures from clinical samples, blood, or milk. However, in certain situations where there is a lack of sufficient facilities, such as advanced biosafety and biosecurity levels (BSL), optimal storage condition for specimens or insufficient time to isolate bacteria, the efficacy of this procedure has been lost. In such circumstances, using alternative tests is given preference. *Brucella* grows slowly, and colonies become visible 3–4 days after culture; however, in some cases, it may take more than two weeks to obtain definitive results [13]. Isolating *Brucella* is a time-consuming, expensive, and hazardous process prone to low sensitivity, leading to increased false negatives. Serological methods are not definitive because not all infected animals produce detectable levels of antibodies, which can result in false-negatives [12]. Several other Gram-negative bacteria, particularly *Yersinia enterocolitica* O: 9, *Escherichia coli* O157:H7, *Salmonella* group N (O: 30), and *Vibrio cholerae* O1, may induce antibody responses in infected animals, leading to false-positive results in serological brucellosis tests [14]. Cross-reactivity between soft *Brucella* and other organisms from different genera has been well-investigated and documented [15]. In dairy animals, *Brucella* replicates in mammary glands and supra-mammary lymph nodes and continuously excretes this intracellular pathogen in milk throughout their lives [16]. Raw milk contains somatic cells, primarily polymorphonuclear leukocytes, macrophages, lymphocytes, and a small percentage of mammary epithelial cells [17]. Although milk contains inhibitors such as fat and protein, it is still possible to extract DNA from milk [18–20]. DNA-based tracking methods have the potential for the rapid, accurate, and efficient detection of *Brucella* in sheep milk. In recent years, studies have been conducted to identify *B. melitensis* in sheep milk using various molecular methods [21–24]. This study employed quantitative polymerase chain reaction (qPCR) using fluorescence-based detection because of its high sensitivity, faster speed, and ability to assess copy numbers compared to conventional PCR [25]. The efficiency of large-scale pathogen screening campaigns can be enhanced by sample pooling methods, which lower the number of tests and save reagents [26]. The main objective of this study was to investigate the ability of qPCR tests to identify *Brucella* bacteria on pooled sheep milk as a noninvasive, efficient, rapid, and cost-effective method at the herd level to reduce the number of tests and precisely categorize positive and negative individuals. Subsequently, the elimination of the source of contamination in the herd will lead to a reduction in negative economic and public health consequences.

Results

A standard curve was plotted to evaluate the performance of the other tests conducted in this study and the prevailing conditions. The generated standard curve had an equation of $y = -3.36X + 34.53$, with an efficiency of 0.986 and an R2 value of 0.9979 (Fig. 1).

Evaluation of the detection limit for bacterial culture revealed that a minimum of 300 CFU of *Brucella melitensis* should be present in each ml of milk to be isolated by microbial culture. When the bacteria were diluted to 200 CFU, only 40% of the cultures tested positive. The results indicated that the presence or absence of CO_2 in the culture medium did not affect the growth of *Brucella melitensis* in milk when using the microbial isolation method.

By performing the qPCR assay on DNA template from serial dilutions of in vitro *Brucella*-contaminated sheep

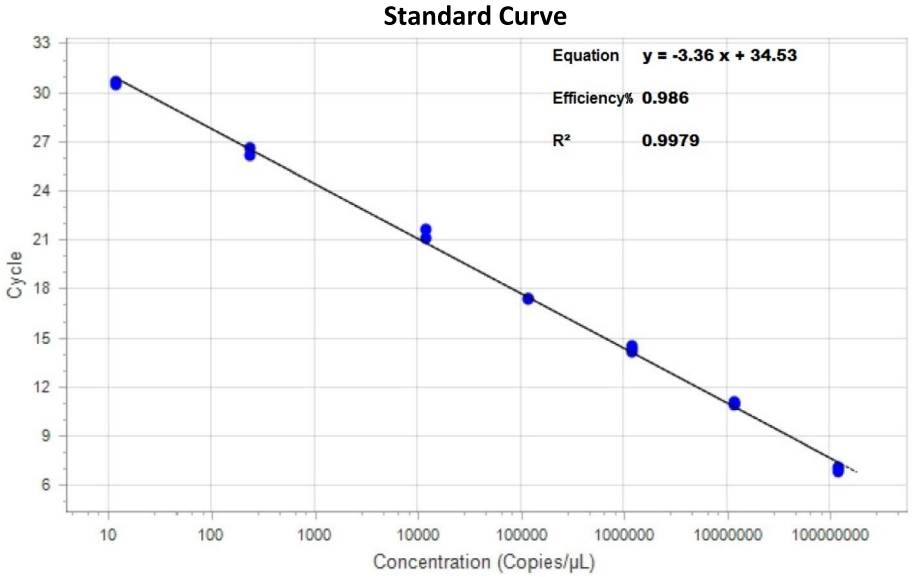

Fig. 1 The standard curve was obtained to detect Brucella DNA using the SYBR Green PCR method, with an efficiency of 98.6%

milk (30, 15, 10, and 5 CFU per 100 µl), the limit of detection for 10 CFU of *Brucella* melitensis organism in each 100 µl of milk was achieved in all five replication. However, when the quantity was reduced to 5 CFU/100 µL, the qPCR assay could not detect *Brucella* DNA in the samples (Table 1).

After examining all 186 samples of the groups, subgroups, and individuals using qPCR, it was shown that from the six groups of 24, four groups (B24, C24, D24, and F24) were positive, and two were negative. From the 12 subgroups of 12-samples, six subgroups (B12a, C12a, C12b, D12a, F12a, and F12b) were positive and six were negative. Of the 24 subgroups of 6, eight (B6b, C6a, C6c, C6d, D6a, F6b, F6c, and F6d) were positive and 16 were negative (Table 2).

Out of the 144 individual samples, nine samples tested positive, while the remaining 135 samples tested negative. By assessing the results of all qPCR tests, it was indicated that none of the subgroups and individual samples in groups A24 and E24, which had negative test results, were positive. In contrast, in groups B24, C24, D24, and F24, which had positive results, at least one subgroup and individual sample tested positive.

Discussion

Brucella bacteria are among the essential abortifacient pathogens in herds, which impose financial losses to the livestock industry and threaten public health, particularly in regions such as the Middle East and the Mediterranean, with the most human cases of brucellosis

Table 1 Limit of detection for bacterial concentrations of 5, 10, 15, and 30 CFU in 100 µl of milk

	Bacterial Count							
	30		**15**		**10**		**5**	
Sample's Number	C_t	N	C_t	N	C_t	N	C_t	N
1	29.96	23	31.13	10	31.94	6	-[b]	0
2	29.51	31[a]	30.77	13	31.43	8	-	0
3	30.31	18	31.35	9	32.38	4	-	0
4	30.15	20	31.22	10	32.22	5	-	0
5	29.85	25	31.96	12	31.58	8	-	0

N Number of detected bacteria, C_t Cycle threshold

[a] A probable error in pipetting led to the detection of more than the initial sample concentration

[b] Lack of detection in this concentration

Table 2 Subdivision of samples into groups and subgroups and the qPCR results

N	Group Name																							
24	A_{24}				B_{24}				C_{24}				D_{24}				E_{24}				F_{24}			
12	A_{12a}		A_{12b}		B_{12a}		B_{12b}		C_{12a}		C_{12b}		D_{12a}		D_{12b}		E_{12a}		E_{12b}		F_{12a}		F_{12b}	
6	A	A	A	A	B	**B**	B	B	**C**	C	**C**	**C**	**D**	D	D	D	E	E	E	E	F	**F**	**F**	**F**
	6a	6b	6c	6d	6a	**6b**	6c	6d	**6a**	6b	**6c**	**6d**	**6a**	6b	6c	6d	6a	6b	6c	6d	6a	**6b**	**6c**	**6d**

Highlighted cells indicate positive qPCR results for the respective groups and subgroups

N Number of pooled samples in each group

[27–30]. Among all *Brucella* species, *B. melitensis* primarily infects sheep as its preferred host [27, 29, 31–33], while a small percentage of sheep brucellosis-related abortions are attributed to *Brucella abortus* [34–36], and *Brucella ovis* [37]. The primers used in the present study identified *Brucella melitensis* and detected *Brucella abortus*, *Brucella ovis*, and several other *Brucella spp*. The positive results obtained in the qPCR assay showed the presence of a minimum of 100 CFU of *Brucella melitensis* organisms per ml of milk. In contrast, the direct culturing method isolated 300 CFU of the same organism. These findings differ from the study conducted by Hamdy et al. in 2002 [21], where the PCR method detected a minimum of 1000 CFU of *Brucella melitensis* in each ml of sterile cow milk. Hinić et al. in 2008 [38], showed that the qPCR test was capable of identifying a minimum of 10 copies of the IS711-based target gene, while Lindahl-Rajala et al. in 2017 [39], obtained results with even lower sensitivity using the same technique and target in cow milk. Possible reasons for these differences could be the use of sterilized sheep milk for dilution, the DNA extraction method employed, the application of a new pair of primers, and the higher sensitivity of the qPCR assay, which may have resulted in less contamination than the PCR assay. Since qPCR does not require post-amplification handling, the results were obtained much faster in less than two hours. In addition, this method's risk of laboratory contamination and false-positive results is less than that of conventional PCR because of the closed tube system [40]. After determining the limit of detection based on the standard curve equation (excluding sample number 2 with a concentration of 300 CFU per ml), the qPCR test did not identify a range of 17 to 60% of inoculated bacteria. Extracting DNA from milk somatic cells is challenging because of the presence of milk fat, which can impede extraction methods [41]. Naturally, the elimination of Brucella bacteria from infected animals occurs intracellularly, but Brucella bacteria are present extracellularly in the inoculated milk samples. It has been reported that a percentage of bacteria present in milk accumulates in the fat layer of milk during gravity separation [42, 43]; therefore, it is possible that a portion of the inoculated *Brucella* bacteria in milk was retained in the fat layer and discarded during the extraction process, which could introduce significant biases in the final PCR assay results. In an experiment conducted by Sun et al. in 2019 [43], three bacterial species were added to bovine milk and separated into fractions. While most bacteria were divided into pellets (73.5–92.6%), a significant proportion remained in the fat layer (7.4–26.5%). The extraction of DNA from milk somatic cells has always been a time-consuming and costly process, accompanied by toxic chemical substances and a high volume of milk (15-50mL) [18–20, 44–47]. In addition, commercial kits for DNA extraction can add significant financial costs. Thus, in the present study, an attempt was made to apply the extraction method described previously by Pokorska et al. 2016 [48], to extract DNA of the Gram-negative bacteria (Brucella), both extra and intra-cellular. This extraction method can be considered practical as a rapid, safe, and cost-effective DNA extraction from sheep milk with a suitable milk volume (10 ml). However, as the quality and quantity of the extracted DNA template were not examined in this part of the study, it cannot be definitively attributed to the related extraction quantity and/or quality.

Screening and diagnostic methods are the main tools for effective epidemiological analyses [48]. In recent years, several studies have been conducted in Iran and other parts of the world to identify and monitor *Brucella* in domestic animal milk using qPCR tests [23, 39, 49, 50]. All the collected samples were examined individually in these studies. The WHO recommends applying the PCR method only to confirm Brucellosis detection in individual samples. It does not recommend herd-level monitoring because of its high cost and time-consuming nature. The present study aimed to decrease the cost of using qPCR for herd-level screening by reducing the number of tests required to identify infected animals by pooling sheep's milk samples. The analysis of the results showed that the 24-sample groups with negative consequences also had negative results in all subgroups and individual samples. In contrast, the 24-sample groups with positive results had at least one positive sample in the corresponding subgroups and individual samples.

Sheep infected with *Brucella melitensis* usually experience only one abortion and give birth to healthy or weak lambs in subsequent pregnancies [51]. These animals can serve as a permanent source of infection within a flock [52]. There are two programs for combating *Brucella melitensis* infection in sheep: 1) control based on mass vaccination and 2) eradication based on tests and slaughtering with or without vaccination. For monitoring purposes, serological tests can be used, or if available, qPCR tests can be used through the milk pooling method after lambing. This approach allows for the identification of infected animals within the flock with fewer tests, in addition to using a noninvasive method. This method should be performed periodically at the flock level, which results in a reduction in economic losses and an improvement in public health.

Conclusion

No serological test alone can examine all epidemiological conditions or animal species. All the tests had limitations, especially for individual animals. It is essential to consider all variables that affect the relationship between the test method and the results with a specific interpretation or diagnostic application. Therefore, validation of the test to understand its characteristics and make decisions about its use for the purpose of the study, epidemiological surveillance, and global trade is crucial. In this regard, the evaluation of diagnostic applications depends not only on the test accuracy but also on the test capacity, technical complexity, and cost-effectiveness. The qPCR-based diagnostic method for detecting *Brucella* in pooled sheep milk at the herd level is a complementary test that can be used following the seropositive cases, as a noninvasive method requiring fewer tests, with lower risks, faster results, and lower costs, without bacterial isolation culture. Although, it should be considered that this test requires milk samples, which are not available all the time, and have an optimal time in which they must be collected to provide an accurate diagnosis.

Methods

Sampling and groups

A total of 144 samples were randomly selected from four individual farms ($n=38$ in each herd) with history of abortion and serological evidence for Brucellosis, located in Khorasan-Razavi County, northeastern Iran. The herds were traditionally managed in groups of 200 to 400 animals and were fed manually supplemented rations in an open-range system. The herds were dual-purpose, managed for both meat and milk production. Vaccination against Brucella was performed only once using Rev1 vaccine, between the ages of 4 to 6 months, prior to breeding. Milk Samples were collected between 2 and 3 months after parturition, and at the end of the lamb weaning time. For each sampling, 30 ml milk from both teats (15 ml from each teat) was collected in a sterile 50 ml tube. Before collecting milk samples, the teats were cleaned with warm water and a disposable towel, and the first milk squirts were discarded. To prevent cross-contamination, the gloves were changed after each sampling. After obtaining specimens under hygienic conditions, they were kept on ice and transferred to the laboratory within a maximum of three hours. To subdivide the individual samples into groups, a volume of 2 ml milk from each sample was randomly pooled into six groups of 24, and each group was divided into pools of 12 and 6, respectively. Finally, 186 milk samples (six pools, $n=24$; 12 pools, $n=12$; and 24 pools, $n=6$, along with 144 individual samples) were subjected to the experiment. After subdivision, the milk samples were stored at −80 °C.

Milk processing and DNA extraction

DNA was isolated from milk samples according to a method previously described by Pokorska et al. 2016 [48]. The DNA precipitate at the end of the extraction process was dissolved in 100 µL TE buffer (pH 8.0, 10 mM Tris, one mM EDTA) and stored at − 20 °C for further examination.

Table 3 Primers used for detection of *Brucella spp*

Primer	Forward	Reverse	Length
qPCR	5′-TCCTCGGTCCAGACA TAG-3′	GCGATGATTTATTCCGTA TCC[a]	142 bp

[a] Since the reverse primer sequence was inverted (5′ to 3′) in different *Brucella* species, the reverse primer movement is not mentioned

Molecular identification

Primer design

To detect most variants of *Brucella*, we applied a pair of primers previously designed by Aminzadeh et al. 2023 [53], for the conserved region of the complete genome sequences of *B.ceti* (NC_022905.1), *B.abortus* (NC_007618.1), *B.melitensis* (NC_003317.1), *B.canis* (NC_010103.1), *B.microti* (NC_013119.1), *B.neotomae* (NZ_UIGH01000001.1), *B.ovis* (NC_009505.1), *B.suis* (NC_004310.3) (Table 3).

DNA amplification

To prepare molecular standards, a conventional PCR test was performed on the extracted DNA from a pure culture of *Brucella melitensis* obtained from the Center of Excellence in Ruminant Abortion and Neonatal Mortality, Ferdowsi University of Mashhad (COE-RANM). After electrophoresis of the PCR product on a 1% agarose gel, the gel fragment containing a 142 bp segment was isolated and extracted using a gel extraction kit (DENA ZIST ASIA®, Mashhad, Iran) and stored at −20 °C. PCR reactions were carried out in 20 µL mixtures containing ten pmol of each primer (Metabion International AG, Planegg, Germany), 10 µL Taq 2×Master Mix Red (Ampliqon A/S, Odense, Denmark), 5µl of template DNA and three µl of UltraPure™ DNase/RNase-Free Distilled water. Amplifications were performed using the following conditions: after an initial denaturation step at 94 °C for 10 min, amplification was carried out with 40 cycles at a melting temperature of 94 °C for 30 s, an annealing temperature of 62 °C for 30 s, and an extension temperature of 72 °C for 30 s, followed by an additional extension at 72 °C for 10 min.

Real-time PCR assay and standard curve generation

All real-time PCR tests in this study were performed using a ten µL mixture containing five µL RealQ Plus 2×Master Mix Green, without ROX (Ampliqon A/S, Odense, Denmark), five pmol of each reverse and forward primer (Metabion International AG, Planegg, Germany), one µL extracted DNA, and three µL UltraPure™ DNase/RNase-Free Distilled water. Amplification and detection were performed using a micro-PCR thermocycler (Applied Biomolecular Systems Co., Australia). The thermocycling process included activation at 95 °C for 15 min, followed by 35 cycles of heating at 95 °C for 30 s and annealing at 60 °C for 30 s. Subsequently, melting curve analysis was conducted between 65 °C and 95 °C. The baseline and threshold were established using the auto baseline and threshold function in the mic-PCR® Software v2.6.4 (Applied Biomolecular Systems Co, Australia). Additionally, in all qPCR tests performed in this study, the samples were tested twice and considered positive if the threshold cycle (Ct) was 35 or less, with a melting temperature of 84.5–85 °C. The purified DNA of molecular standard was quantified using a spectrophotometer (Biophotometer® M6131, Eppendorf AG, 22331 Hamburg, Germany), and based on the molecular weight of the 142 bp fragment, the number of present pieces was calculated by online software of copy number calculator (www.technologynetworks.com). A standard curve was generated using tenfold-serial dilutions of the molecular standard and threshold cycle (Ct) values for absolute quantification. Each dilution was tested three times, and the obtained Ct values and corresponding concentrations were used to generate a standard curve.

The minimum detectable concentration of Brucella organism in inoculated milk using culture medium and qPCR

To determine the limit of detection for microbial culture, five frozen samples of *Brucella melitensis* (obtained from COE-RANM) were initially inoculated into *Brucella* broth medium (Merck, Darmstadt**,** Germany), and after 72 h, the bacterial count in the medium was determined using a spectrophotometer (Biophotometer® M6131, Eppendorf AG, 22,331 Hamburg, Germany). Then, 1 ml of medium containing a specific number of bacteria was mixed with 9 ml of *Brucella*-free sheep milk and different dilutions of 600, 300, 200, and 100 CFU/ml were simultaneously prepared from each of them, and each dilution was inoculated onto two plates containing a 5% blood agar culture medium. Each dish was placed in either an aerobic atmosphere or an atmosphere containing 8–10% CO_2 (Microbiology Anaerocult c®, Merck, Darmstadt**,** Germany) and incubated at 37 °C for 72 h. After 72 h, if bacterial growth was observed, the culture was considered positive. The plates were incubated for over 72 h if no growth was observed. If there was no growth in the third time interval, it was considered a negative culture at the respective dilution.

To assess the minimum detectable concentration of Brucella in milk by qPCR assay, dilutions of 300, 150, 100, and 50 CFU/ml in *Brucella*-free sheep milk were prepared, and after DNA extraction, the resulting products were subjected to qPCR assay.

Determining the optimal size for pooled milk lots

To optimize the number of samples per pool, after optimizing the real-time PCR a group of random seropositive sheep milk samples referred to the COE-RANM were tested using qPCR (unpublished data). It was determined that these positive samples had at least 3000 CFU of Brucella /mL of milk. Based on our test threshold, which is 100 CFU/mL, we can calculate that each sample has the ability to be diluted 30 times. To ensure greater accuracy,

a 20% margin of error was taken into, resulting in a final number of 24. It means, a maximum number of 24 samples were considered to be pooled for each group. Moreover, to ensure the accuracy of the pooling each pool of 24 (6 pools with 24 samples in each) was divided and tested in corresponding pools of 12(12 pools with 12 samples in each) and 6 (24 pools with 6 samples in each), and even each individual sample was tested separately. therefore, no individual positive sample will be missed and all pools with different number of samples will be considered and compared.

Data analysis

All the real-time PCR assessments, the baseline and threshold were set using the mic-PCR® Software v2.6.4 features (Applied Biomolecular Systems Co., Australia).

Abbreviations

COE-RANM	Center of Excellence in Ruminant Abortion and Neonatal Mortality
CFU	Colony Forming Unit
WHO	World Health Organization
Ct	Threshold Cycle

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Authors' contributions

Mirshokraei.P. contributed to the design Rahmani.Hk., Hashemi.K., Mirshokraei.P. conceive of the experiment Aminzadeh. M.J., Khaleghnia.N. experimented Azizadeh M. statistical analysis Aminzadeh. M.J., Khaleghnia.N. wrote the manuscript Mirshokraei. P revision of the manuscript.

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Data availability

Data is provided within the supplementary information files. All the research processes carried out on animals were based on the ethical guidelines of research from Ferdowsi University of Mashhad (ethics code: IR.UM.REC.1401.063).
All the research processes carried out on animals were based on the ethical guidelines of research from Ferdowsi University of Mashhad (ethics code: IR.UM.REC.1401.063).

Declarations

Ethics approval and consent to participate

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to, and the appropriate ethical review committee approval has been received (research ethics committee of Ferdowsi University of Mashhad: IR.UM.REC.1401.063). The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes. Moreover, the informed consent forms were properly obtained from the owners for all the research processes that were carried out on animals.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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